Sleep in Mice with Non-Functional Growth Hormone Releasing Hormone Receptors

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Running Head: Sleep in mice with non-functional GHRH-receptor

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ABSTRACT

The role of the somatotropic axis in sleep regulation was studied by using the lit/lit mouse with non-functional growth hormone (GH)-releasing hormone (GHRH) receptors (GHRH-Rs) and control heterozygous C57BL/6J mice, which have a normal phenotype. During the light period, the lit/lit mice displayed significantly less spontaneous rapid eye movement sleep (REMS) and non-REMS (NREMS) than the controls. Intraperitoneal (ip) injection of GHRH (50 µg/kg) failed to promote sleep in the lit/lit mice whereas it enhanced NREMS in the heterozygous mice. Subcutaneous infusion of GH replacement stimulated weight gain, increased the concentration of plasma insulin-like growth factor-1 (IGF-1), and normalized REMS, but failed to restore normal NREMS in the lit/lit mice. The NREMS response to a 4-h sleep deprivation was attenuated in the lit/lit mice. In control mice, ip injection of ghrelin (400 µg/kg) elicited GH secretion and promoted NREMS, and ip administration of the somatostatin analog, octreotide (OCT, 200 µg/kg) inhibited sleep. In contrast, these responses were missing in the lit/lit mice. The results suggest that GH promotes REMS whereas GHRH stimulates NREMS via central GHRH-Rs and that GHRH is involved in the mediation of the sleep effects of ghrelin and somatostatin.
INTRODUCTION

Regulation of sleep and the somatotropic axis are intimately related. Deep non-rapid eye movement sleep (NREMS) is associated with growth hormone (GH) secretion (reviewed in 53). The somatotropic axis hormones, GH-releasing hormone (GHRH), GH, insulin-like growth factor-1 (IGF-1), and somatostatin are capable of modulating sleep. The sleep-promoting activity of GHRH is documented in rats and rabbits (11,36) and humans (21,28,45). Independently from GHRH, GH may increase the intensity of NREMS (2) and the duration of rapid eye movement sleep (REMS) (10,30,46). IGF-1 may promote NREMS (39), and somatostatin may stimulate REMS (7). In addition, acute rises in somatostatin, GH, and IGF-1 can suppress sleep via negative feed back inhibition of GHRH (reviewed in 40). GHRH and the GHRH receptor (GHRH-R) genes are contained within the genomic regions implicated in homeostatic sleep regulation (12) and the sleep responses to viral influenza infection (52) in mice. Chronic hypoactivity of the somatotropic axis is associated with sleep alterations. Thus, REMS and the time spent in deep NREMS may decrease though total sleep time may increase in humans with decreased GH production (reviewed in 2). Transgenic mice with GHRH deficiency (TH-hGH mice) have decreased NREMS (57). Spontaneous NREMS and REMS are lowered in the mutant dw/dw rat (37) which has a defect in GHRH-R signaling. In animals with decreased GHRH synthesis or a defect in GHRH-R signaling, however, the physiological stimulus of pituitary GH synthesis and release is absent, and concentrations of both GH and IGF-1 are low in the plasma resulting in a dwarf phenotype. Therefore, the significance of the individual hormones in the sleep deficit is difficult to clearly determine.
The aim of the current experiments was to use the \textit{lit/lit} mouse to study the role of GHRH in sleep regulation. The \textit{lit/lit} mouse bears a point mutation in the GHRH-R gene resulting in a loss of receptor function (15,25), a model of genetic GHRH-R defects in humans (54). By using the \textit{lit/lit} mouse, we report that chronic GH infusion normalizes REMS whereas the NREMS deficiency persists in the chronic hypofunction of the somatotropic axis suggesting that these sleep alterations are linked to GH/IGF-1 and GHRH deficiencies, respectively. The \textit{lit/lit} mice fail to exhibit sleep responses if given GHRH, the somatostatin analog, octreotide (OCT), or ghrelin, a hormone acting on the GH-secretagogue (GHS) receptor (\textit{GHS-R}) (22) showing that these sleep responses are mediated via GHRH actions on GHRH-Rs.

\textbf{METHODS}

\textit{Animals.} Heterozygous male C57BL/6 (C57BL/6J-Ghrhr-lit-/+), and homozygous male \textit{lit/lit} mice (n=56, each) were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were between 3 and 4 months of age at the time of the experiments. The heterozygous controls weighed 24.1 \pm 0.69 g, the \textit{lit/lit} mice weighed 11.4 \pm 0.31 g (47.2 \% of the body weight of the controls). The difference in body weight was significant between the two groups (Student t-test, p<0.0001). The animals were housed individually in Plexiglas® cages placed in environmental chambers on a 12-12 h light-dark cycle, at 30°C ambient temperature. Food and water were continuously available.

\textit{Surgery.} The mice were anesthetized with ketamine-xylazine (87 and 13 mg/kg, respectively). Stainless steel electrodes were placed on the top of the dura mater over the frontal and parietal lobes and the cerebellum. These electrodes were used to record the electroencephalogram (EEG).
Two stainless steel wires were implanted into the dorsal neck muscles to record the electromyogram (EMG). The EEG and EMG electrodes were connected to a pedestal (0.4 g) implanted on the top of the skull. The surgeries were performed 7 days before recordings began.

*Recording of sleep-wake activity.* The mice were connected to light-weight recording cables and habituated to the experimental conditions during recovery. The cables were attached to commutators which were connected to amplifiers. The signals were digitized (128-Hz sampling rate) and collected by a computer and stored on compact discs. Power density spectra (0.25-40.0 Hz in 0.5-Hz bands) were calculated on-line for consecutive 10-s EEG epochs and stored with the EEG and EMG signals. For scoring, the EEG and EMG signals and the power density spectra were restored on the computer screen. The states of vigilance were determined for 10-s epochs by the usual criteria as NREMS (high-amplitude EEG slow waves and low-tone muscle activity), REMS (highly regular theta EEG activity and loss of muscle tone with occasional twitches), and wakefulness (EEG activities similar to, but often less regular and with lower amplitude than, those in REMS and high EMG activity). The percentage of the time spent in each state of vigilance for 1-h periods was determined. The power values for the 0.5 to 4.0 Hz (delta) frequency range were integrated. The mean of these integrated values were calculated for uninterrupted periods of artifact-free NREMS, and used as an index of EEG slow wave activity (SWA) during NREMS to characterize sleep intensity (depth of sleep) in each recording hour. When studying the peptide effects, sleep latency was also determined. Sleep latency was defined as the time prior to the first occurrence of 30-s continuous NREMS episode measured from the onset of the light period (after OCT administration) or dark period (after GHRH and ghrelin administration).
Experimental schedule. Starting at light onset, the mice (n=26 heterozygous, and n=25 lit/lit mice) were recorded from for two consecutive days to obtain baseline values of spontaneous sleep-wake activity. On the third experimental day in 13 of the control and 13 of the lit/lit mice, sleep deprivation (SD) started at light onset and lasted for 4 hours. The EEG and EMG were recorded throughout SD, and during the remaining 8 h of the light period, and for 11 h during the subsequent dark cycle (collected data were backed up sometime during the last hour of the dark period and the records in this hour were discarded). SD was performed by gentle handling while the mice stayed in their home cage; whenever behavioral or EEG signs of sleep were observed, the mice were aroused by knocking on the cage or lightly touching them. It is assumed that stress is not a major factor determining the sleep response to SD, at least when SD is performed by gentle handling (18), though sleep loss itself may represent a stressor and may alter hypothalamo-pituitary-adrenal functions (29).

The mice, which were used in the GH replacement experiment, were subcutaneously implanted with osmotic mini-pumps (ALZET 1002, 0.25 µl/h for 14 days; Durect Corporation, Cupertino, CA) in the back (weight after loading: 0.5 g). The minipumps were filled with mouse GH (mGH) in physiological saline delivering 11 µg mGH per day in 4 lit/lit mice. Since animal GH preparations are highly purified extracts obtained from the pituitary gland, for infusing a larger GH dose, rat GH (rGH) was used because it was available in larger quantities. Rat GH exerts actions closely resembling, if not identical to those of endogenous mGH in mice (3). Minipumps delivering 24 µg rGH per day were implanted in 8 lit/lit mice. This dose of GH was previously reported as biologically active when infused from minipumps in lit/lit mice (33). A
group of heterozygous mice (n=12) received osmotic pumps delivering physiological saline at the same rate as the GH delivery in the lit/lit mice. Spontaneous sleep-wake activity of the mice implanted with the minipumps was recorded on day 8 and/or 9 of the infusion. The mGH and rGH preparations were for in vivo use, and they were gifts from Dr. A.F. Parlow of The National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases (NHPP-NIDDK, Bethesda, MD).

GHRH and ghrelin were anticipated to promote sleep, and therefore the sleep response was studied during the active (dark) phase of the day when mice exhibit little spontaneous sleep. In contrast, OCT suppresses sleep (16), and thus OCT was injected prior to the rest cycle, i.e. the light period. GHRH and ghrelin (Bachem/Peninsula Laboratories, Inc. San Carlos, CA) were dissolved in physiological saline. The peptides (5 µg/kg GHRH, n=14 heterozygous mice and n=13 lit/lit mice; 400 µg/kg ghrelin, n=11 heterozygous mice and n=11 lit/lit mice) were intraperitoneally (ip) injected in a volume of 0.1 ml/10 g body weight. The injections were timed to 10 min prior to dark onset. Physiological saline was administered on the baseline day. The OCT (Sandostatin injection, Novartis Pharma, Basel, Switzerland) dose was 200 µg/kg in a volume of 0.1 ml/10 g body weight (n=11 heterozygous mice and n=12 lit/lit mice). OCT was ip injected 10 min prior to the onset of the light period, and the mice stayed in light during this 10 min. For baseline injections, the vehicle of OCT containing lactate and mannitol was used; Novartis Pharma donated this solution. In the experiments with ghrelin and OCT, the mice were scored for bouts of eating and drinking, respectively, during the first 10 min postinjection. The eating or drinking response was considered positive if a mouse produced at least one bout of this behavior. After injections, sleep-wake activity was recorded for 12 h during the dark period.
(GHRH, ghrelin) or light period (OCT). The sleep responses, however, occurred within 1 to 3 hours postinjection. Therefore, only data from the first 4 hours of the 12 h records are reported herein. In each group, the order of the baseline (vehicle) and experimental (peptide) day varied, and approximately half of the groups were injected with the vehicle and half of the groups with the peptide on the first day. Some of the mice tested with GHRH were previously used in the SD experiments. In this case, one week elapsed between these experiments. Some mice were tested with both ghrelin and OCT; there was at least one day off between these tests.

**Ghrelin-induced GH secretion.** The effects of an ip injection of 400 µg/kg ghrelin on plasma GH concentration was studied in 7 lit/lit and 8 heterozygous mice. To obtain control values, physiological saline was injected to 10 lit/lit and 10 heterozygous mice. The mice were decapitated 15 min after injections and the trunk blood was collected. The blood was centrifuged and the plasma was stored at –20 °C until the radioimmunoassay. The immunoreagents for determining plasma GH were provided by NHPP-NIDDK. GH was measured in duplicate, the intra-assay coefficient of variation was less than 7%.

**Effects of GH infusion on plasma IGF-1 concentration.** Since IGF-1 is a GH-dependent hormone in the plasma, IGF-1 concentration indicates the efficacy of the GH delivery by the osmotic pumps. In separate experiments, another 7 lit/lit mice were implanted with minipumps delivering 24 µg rGH per day, and 8 heterozygous mice were provided with minipumps infusing physiological saline. These mice were sacrificed on day 7 of the infusion to determine IGF-1 concentrations in the plasma of the trunk blood. The plasma of 2 lit/lit mice, which did not receive GH, was used to estimate baseline IGF-1 concentration. The blood samples were
centrifuged and the plasma was stored at – 20 °C. IGF-1 was measured in duplicates by means of rat IGF-1 enzyme immunoassay kit (EIA kit, Diagnostic Systems Laboratories, Inc., Webster, TX). The antiserum crossreacts with mouse IGF-1, and does not bind to IGF-2 (rat, human).

_Determination of the point mutation in intracerebral GHRH-R._

The hypothalamus plus preoptic region (landmarks: frontal edge of the optic chiasma, lateral sulci, mammillary bodies, and a depth of 1 mm) and the pituitary gland were harvested from two _lit/lit_ mice and two heterozygous mice. Total RNA was isolated from hypothalamus and pituitary using GibcoBRL’s TRIzol reagent. The RNA was then subsequently DNase-treated with RNase-free DNase from Ambion (Austin, TX). Using a high fidelity enzyme in the TITAN one-tube RT-PCR system from Boehringer Mannheim (Germany). GHRH-R mRNA was amplified from 1 µg RNA; the primers used were sense 5’ (cggcagaggggaccaacaac) and antisense 5’ (cacaggcaagccacagggtatg).

The RT-PCR product was electrophoresed on a 1% agarose ethidium bromide gel, and purified by MinElute Gel Extraction kit from Qiagen (Valencia, CA). Gel isolated DNA was then subsequently amplified for nucleotide sequencing by the Department of Biochemistry from Washington State University.

_Statistics._ The results are provided as mean values ± SE. Sleep parameters on the two baseline days or the two days after GH infusion were averaged for each mouse. Two-way ANOVA was used to compare hourly values of NREMS, REMS and SWA among the various groups, and between baseline and experimental day in one group. Group effects (independent samples; e.g.
heterozygous vs. \textit{lit/lit} mice) or treatment effects (repeated measures; e.g. sleep before and after GH infusion, sleep on the baseline day and after GHRH, ghrelin, or OCT), and time effects (repeated measures) were the two factors of the ANOVA. The time blocks corresponded to the 12-h light and dark periods for spontaneous sleep, the 8-h postdeprivation light period and 11 h at night after SD, and 4-h postinjection periods after administration of GHRH, ghrelin, and OCT. Since variations in sleep parameters with time are well known, only the group or treatment effects are reported herein. Because mice exhibit periods of wakefulness longer than one hour at night, hourly values of SWA during NREMS could not be analyzed by ANOVA. Instead, SWA values during NREMS were averaged for the 12-h dark period and these values were used for comparison. Sleep parameters in hour 1 postinjection after GHRH, ghrelin, and OCT were compared to baseline values after vehicles by means of the paired t-test. Also, the changes in body weight in response to GH were evaluated by means of paired t-test. Mean duration of NREMS and REMS bouts was calculated for 12 h periods and compared by means of Student t-test between heterozygous and \textit{lit/lit} mice. Student t-test was used for comparisons of hormone concentrations, body weights, and SWA at night between groups. When conditions for parametric tests were not fulfilled the appropriate non-parametric tests were used (Mann-Whitney test or Wilcoxon test); together with the level of significance, the statistical tests are indicated throughout text. An $\alpha$ level of $p < 0.05$ was considered to be significant in all tests.

These experiments are consistent with the guiding principles for research on animals issued by the American Physiological Society (1).

\textbf{RESULTS}
**Mutation of hypothalamic GHRH-R**

The nucleotide sequence of the hypothalamic GHRH-R obtained from the *lit/lit* mice differed by one base (a guanine was substituted for an adenine) thereby leading to an altered codon 60 changing from aspartic acid to glycine in the *lit/lit* mice. These results confirm those previously reported for the pituitary GHRH-R in the *lit/lit* mice (15).

**Spontaneous sleep**

The control heterozygous mice and the *lit/lit* mice exhibited a normal diurnal rhythm of sleep with more NREMS and REMS during the light period than at night (Fig. 1). The *lit/lit* mice spent significantly less time in NREMS than the heterozygous mice during the light period [F(1,49)=60.22, p<0.0001]. The NREMS deficit was 13.2 % of the recording time, yielding 95 min less NREMS in the *lit/lit* mice than in the controls during the 12 hour light period. This reduction in duration of daytime NREMS resulted from large and significant reduction in the mean duration of the NREMS episodes (1.30 ± 0.03 min in the *lit/lit* mice vs. 2.02 ± 0.06 min in the controls, Mann-Whitney test, p<0.0001). The number of the NREMS episodes was slightly, but significantly higher in the *lit/lit* mice than in the heterozygous mice (206 ± 7.3 vs. 185 ± 5.3, p<0.05, Student t-test). NREMS tended to decrease at night but this difference did not reach the level of statistical significance. The *lit/lit* mice had significantly less REMS than the controls during the light period [-2.25 % recording time, corresponding to 16.2 min in 12 h light period; F(1,49)=27.09, p<0.0001]. The REMS deficit resulted from a significantly decreased REMS episode frequency (controls: 44.2 ± 1.12; *lit/lit* mice: 32.7 ± 1.92, Mann-Whitney test, p<0.001) whereas the duration of the REMS episodes was normal in *lit/lit* mice (1.5 ± 0.02 min and 1.5 ± 0.04 min in the heterozygous and *lit/lit* mice, respectively). REMS was not altered at night.
The EEG SWA during NREMS in both mouse strains was relatively high after light onset, declined during the day and rose during the dark period. The interindividual variability in EEG SWA was high and significant differences between the heterozygous and lit/lit mice could not be detected. The nocturnal rise in EEG SWA tended to be smaller in the lit/lit mice than in the controls because the mean NREMS-associated power in the delta frequency range was significantly higher at night than during the day in the heterozygous mice (Wilcoxon-test, p=0.001) whereas mean power values did not differ between day and night in the lit/lit mice. To reduce individual variability, deviation from a 24-h mean EEG SWA was calculated for each hour and each mouse. Comparisons of these values failed to reveal significant variations among the groups. The ratio of delta power to total power during NREMS was determined in the light (heterozygous: 0.49 ± 0.01, lit/lit 0.48 ± 0.01) and dark periods (heterozygous: 0.52 ± 0.01, lit/lit 0.48 ± 0.01), and the values did not differ between groups though the delta ratio tended to be higher in the controls than in the lit/lit mice at night. Finally, powers in the other frequency ranges also did not differ between the two groups (controls and lit/lit mice, 4.5-8.0 Hz: 932 ± 101 and 904 ± 68 µV²; 8.5 – 12.0 Hz: 394 ± 42 and 413 ± 31 µV²; 12.5-16.0 Hz: 159 ± 17 and 150 ± 13 µV²; 16.5-20 Hz: 59 ± 7 and 51 ± 4 µV²).

**Effects of GH Replacement**

After 1 week of GH infusion, the weight gain of the lit/lit mice (+1.9 ± 0.22 g) was significantly higher than the weight gain of heterozygous mice infused with physiological saline (+0.8 ± 0.23, Student t-test, p<0.005). The weight gain may depend on GH dose for it was +2.11 ± 0.22 g in the mice infused with 24 µg rGH/day in contrast with the weight gain of +1.0 ± 0.47 g in the
mice which received 11 μg mGH/day but the n=4 sample size for the lower dose of GH was too low for statistical comparison.

The mean concentration of IGF-1 was 389.3 ± 15.31 ng/ml in the heterozygous mice infused with physiological saline. The plasma IGF-1 concentrations were 24.95 and 27.80 ng/ml in two untreated lit/lit mice. Thus the IGF-1 concentration in the lit/lit mice was about 1/15th of the control value. Plasma IGF-1 rose to a mean of 158.1 ± 20.1 ng/ml (min.: 75.6; max.: 210.6 ng/ml) after one week of infusion of 24 μg rGH/day, indicating that the treatment was effective though IGF-1 concentration remained below normal.

Irrespective of the GH dose, GH infusion failed to alter NREMS in the lit/lit mice (Fig. 1). In contrast, REMS increased in the lit/lit mice receiving chronic GH administration. The REMS response to GH was only slightly stronger after the larger GH dose than after the smaller dose (+13 ± 3.2 min vs. +11 ± 3.4 min in the 12- h light period), and the sample size did not allow statistical comparisons between the doses. In Fig. 1 and in the statistics, the mice infused with the low and high GH doses were pooled. When comparing the duration of REMS in the lit/lit mice before and after GH treatment, the increases in REMS were significant during the light period [F(1,11)=23.12, p<0.001]. After GH infusion, the statistical difference in REMS between the heterozygous mice and the lit/lit mice disappeared. REMS in lit/lit mice at night was not altered by GH administration.

After GH infusion, the nocturnal rise in EEG SWA during NREMS became significant (Wilcoxon test, p<0.001). The ratio of delta power to total power during NREMS was the same
as in the heterozygous mice, i.e. slightly higher than in the lit/lit mice without GH infusion. The other power parameters, including the power in the various EEG frequency ranges, did not differ from values in the heterozygous or lit/lit mice without GH infusion.

Sleep deprivation

The increases in the time spent in NREMS were small after SD; the most obvious change occurred in hour 2 after SD in both groups of mice (Fig. 2). In control mice, however, small increases in NREMS occurred for 6 hours, and the changes were significant calculated for the 8 postdeprivation hours of the light period [+3.3 ± 0.85 % recording time, i.e. 24 min in 8 h; treatment effect: F(1,12)=13.79, p<0.01; treatment x time interaction: F(7,84)=3.69, p<0.005]. Also, significant, albeit small, increases in NREMS were observed during the subsequent dark period [+3.3 ± 1.31 % recording time; F(1,12)=5.85, p<0.05] in the control mice. In contrast, statistically significant alterations failed to occur in NREMS in the lit/lit mice during the light period (-1.2 ± 1.95 % recording time in 8 h) or the dark period (0.0 ± 0.86 % recording time).

Direct comparisons of the magnitude of changes in NREMS between the controls and the lit/lit mice suggested that NREMS responses tended to be higher in the controls than in the lit/lit mice during the day [F(1,24)=4.128, p=0.05] and at night [F(1,24)=3.989, p=0.06].

The time spent in REMS increased during the last hours of the light period after SD in both groups of mice (Fig. 2). Calculated for the 8 postdeprivation hours in the light period, these changes did not reach the level of statistical significance in either group though the SD-induced variations in REMS could be detected in the significant treatment x time interactions [heterozygous mice: F(7,84)=9.02, p<0.0001 and lit/lit mice: F(7,84)=8.574, p<0.0001]. REMS,
however, increased significantly at night in both the heterozygous [+2.1 ± 0.28 % recording time; F(1,12)=51.88, p<0.0001] and the lit/lit mice [+1.74 ± 0.40 % recording time; F(1,12)=17.852, p<0.005], and these changes did not differ between the two groups.

In heterozygous mice, a prominent EEG SWA response was elicited by SD (Fig. 2); EEG SWA during NREMS was about 50% higher in hour 1 after SD. SD-enhanced EEG SWA declined rapidly during postdeprivation hours 2-4 and was below baseline during the subsequent dark period. The increases in EEG SWA were significant [F(1,12)=33.5, p<0.0001] and varied with time [treatment x time interaction, F(7,84)=58.3, p<0.0001] during the light period after SD, and the suppression of EEG SWA was also significant at night (paired t-test, p<0.05). In the lit/lit mice, SD-induced enhancements in EEG SWA were also significant during the light period [F(1,12)=24.6, p<0.001; interaction: F(7,84)=28.0, p<0.0001], and did not differ from those in the controls. The suppression in EEG SWA at night after SD did not reach the level of statistical significance in the lit/lit mice.

Effects of OCT

The amount of NREMS was between 30-40 % recording time in undisturbed mice during the first hour of the light period (Figs. 1 and 2). As indicated by the low amount of sleep in hour 1 the ip injection of the vehicle at light onset was stressful for the mice in both groups (Fig. 3). In the heterozygous mice, injection of OCT significantly delayed sleep onset (32.1 ± 2.5 min after vehicle, and 52.2 ± 3.9 min after OCT, paired t-test, p<0.001), and decreased the time spent in NREMS in hour 1 (paired t-test, p<0.05). In the OCT-treated control mice, NREMS time returned to normal in hour 2. EEG SWA during NREMS increased significantly in hour 2, remained high in hours 3 and 4, then returned to baseline in hour 5 (not shown). These
enhancements in EEG SWA were statistically significant calculated for the 2-4 hour time period \[F(1,10)=20.09, p<0.005\]. REMS did not change after OCT treatment in control mice (not shown). In contrast, sleep was not altered after OCT in the \textit{lit/lit} mice. Each heterozygous and \textit{lit/lit} mouse displayed at least one bout of drinking during 10 min after administration of 200 \(\mu\)g/kg OCT whereas drinking never occurred after injection of the vehicle.

\textit{Effects of GHRH}

GHRH enhanced NREMS in the heterozygous mice (Fig. 3). The latency of sleep onset decreased significantly (39.5 ± 4.3 min after physiological saline, and 27.5 ± 3.2 min after GHRH, paired t-test, \(p<0.05\)), and the time spent in NREMS increased significantly in hour 1 postinjection (paired t-test, \(p<0.0005\)). A second large increase in NREMS time occurred in hour 3. These changes in NREMS were statistically significant when calculated for the 4-h postinjection period \[F(1,13)=30.48, p<0.0001\]. EEG SWA during NREMS and REMS were not altered after injection of GHRH (not shown). In contrast, in the \textit{lit/lit} mice GHRH failed to alter latency to sleep onset (41.8 ± 7.3 min after physiological saline, and 39.6 ± 7.2 after GHRH) or any other sleep parameter measured in this study.

\textit{Effects of ghrelin}

The mean plasma GH concentrations were 20.5 ± 5.71 ng/ml and 7.21 ± 1.1 ng/ml in heterozygous and \textit{lit/lit} mice, respectively, 15 min after ip injection of physiological saline. The difference between the two groups was statistically significant (Mann-Whitney test, \(p<0.05\)). In response to ip injection of 400 \(\mu\)g/kg ghrelin, the GH concentration in the plasma exceeded 128 ng/ml, the top value of the calibration curve, in every control mouse. In the \textit{lit/lit} mice, after
ghrelin injection, the plasma GH concentration was 8.13 ± 2.92 ng/ml, and this value did not differ from the baseline value in these mice.

In the heterozygous mice, ghrelin induced a significant decrease in sleep latency (45.0 ± 6.3 min after saline and 17.2 ± 2.2 min after ghrelin, paired t-test, p<0.001), and a significant increase in NREMS in hour 1 (paired t-test, P<0.05) (Fig. 3). Sleep time, however, returned to baseline thereafter, and the changes in NREMS were not significant when calculated for 4 hours. REMS and EEG SWA were not altered after injection of ghrelin (not shown). In the lit/lit mice, ghrelin had no effect on sleep latency (43.2 ± 13.7 min after saline, and 39.8 ± 9.1 min after ghrelin), duration of NREMS or REMS, or EEG SWA. Bouts of eating were noted in each control mouse and in all but one lit/lit mouse during the initial 10 min after the injection of ghrelin.

DISCUSSION

The experiments were designed with the anticipation that studies on the lit/lit mice would promote our understanding of the role of the somatotropic axis hormones in sleep regulation. The reduction in spontaneous NREMS in the lit/lit mice as reported here is also characteristic of the TH-hGH transgenic mice which have decreased GHRH production (57), and of dwarf (dw/dw) rats which have a defect in GHRH receptor signaling (37). One novelty of the current study is that GH replacement for 8-9 days restores REMS whereas it fails to increase NREMS time in the lit/lit mouse. Although continuous GH delivery does not mimic physiological pulsatile GH secretion (26) it is effective (19) as evidenced in our experiments by the enhanced GH values and plasma IGF-1 concentrations and the weight gain in the lit/lit mice. The differences in the REMS and NREMS responses to GH infusion suggest that the decreases in NREMS are predominantly
independent of GH/IGF-1 whereas the GH deficiency may have a major role in the alterations in REMS in the lit/lit mice. Lit/lit mice tended to display less intense SWA than the control mice particularly during NREMS in the dark period and GH replacement normalized delta power. Although the alterations in the lit/lit mice were only at the border of statistical significance reduced SWA is in agreement with some observations in GH deficient humans (2) and may indicate a role of GH in determining delta power. The REMS-promoting activity of GH (and stimulation of delta power) might be associated with some intracerebral metabolic action (reviewed in 35) for the REMS response to an acute GH injection is blocked by inhibitors of protein synthesis (10).

There is a great variability among mice strains in the effects of SD on NREMS duration (12,18). Calculated in percent recording time, small, 3.6 %, increases in NREMS time were reported during 6 h recovery after 4-h SD in C57BL/6J mice and these changes did not reach the level of statistical significance (18). The increment in NREMS time was in fact small albeit consistent across several hours in both the light and the dark periods in the heterozygous mice. Apart from a brief enhancement in NREMS in hour 2 after SD, this response did not occur in the lit/lit mice. In contrast, both control and lit/lit mice exhibited enhanced EEG SWA after SD. This is unexpected because the SD-induced enhancements in EEG SWA are greatly attenuated in the dw/dw rats (37). In mice, a 4-h SD is suggested to elicit maximal EEG SWA responses (18), and perhaps this amount of SD is not suitable for the demonstration of small differences in responsiveness.
The deficit in GHRH signaling itself is the likely candidate for the decreases in spontaneous NREMS and for the reduced NREMS response to SD in the lit/lit mice. In fact, the NREMS-promoting activity of GHRH does not require GH (38); instead, this GHRH action is mediated by the hypothalamus / preoptic region (58). These observations imply that GHRH-Rs are expressed by neurons in the hypothalamus, and that these receptors are affected by the genetic mutation in the lit/lit mouse. The presence of GHRH-R mRNA was previously described in the rat hypothalamus (47). Current results show that GHRH-R mRNA is also expressed in the hypothalamus / preoptic region of the mouse. The reported point mutation in pituitary GHRH-R (15,25) was demonstrated in the hypothalamic GHRH-R suggesting that central GHRHergic activity is abolished the same way as GHRH actions in the pituitary.

That the NREMS-promoting activity is a specific effect of GHRH on GHRH-Rs is demonstrated by the failure of lit/lit mice to respond to GHRH. To our knowledge, the current report is the first description of the effects of GHRH on sleep in mice. GHRH strongly increased the duration of NREMS but, in contrast to the findings in previously studied species, it failed to increase EEG SWA during NREMS in heterozygous mice. However, the sleep response to an ip injection of GHRH at the onset of the active period in the mouse might not be comparable to the effects of systemically injected GHRH during the rest period in humans and rats, or to intracerebral administration of GHRH during the active period to the rat (reviewed in 40).

Somatotropic deficiency could result in developmental alterations causing permanent sleep deficits. Poor maturation of neuronal networks may occur in GH deficiency including an underdevelopment of the suprachiasmatic nucleus and the pineal gland which may alter circadian
rhythms (34). Although changes in behavior or diurnal rhythms were not obvious in our experiments a contribution of developmental changes to NREMS alterations in the \textit{lit/lit} mice currently cannot be excluded.

Ghrelin promoted NREMS and elicited GH secretion in the heterozygous mice. GHS receptors are expressed both in the pituitary somatotroph cells and in the hypothalamus (31,44), and therefore, GHSs may elicit GH secretion through both sites of action. It seems, however, that stimulation of GH secretion by GHSs requires intact GHRHergic activity (5,27,41) in part because GHRH mediates the effect of GHSs (8,48), and in part because pituitary GHS receptors are down-regulated in the absence of GHRH (20). Stimulation of GHRH-containing neurons and inhibition of somatostatinergic neurons, another proposed action of GHSs (51), make ghrelin a candidate as a sleep-promoting peptide. GHSs, however, elicit feeding (56), and stimulate the CRH/vasopressin-ACTH axis (23,49), and these effects might not be compatible with sleep. In humans, synthetic GHSs have no effects (32) or enhance NREMS (6,13), and ghrelin promotes slow wave sleep (55). Tolle et al. (50) injected ghrelin three times per day to rats, and found inhibition of sleep during 30 min postinjection coinciding with induced eating. Although not analyzed statistically, enhancements in NREMS time could be observed during the subsequent 10-min time blocks in the published figure depicting NREMS. In addition, Tolle et al. (50) reported decreases in REMS in the 9 h observation period during which ghrelin pulses were delivered. Alterations in REMS did not occur in our experiments but the mice were recorded at night when spontaneous REMS time was already low. Although our results provide evidence for the NREMS-promoting capacity of ghrelin the efficacy of ghrelin and GHRH cannot be compared without determining dose-response relationships for both peptides. In the \textit{lit/lit} mice,
ghrelin failed to stimulate NREMS and GH secretion while it continued to elicit feeding. This suggests, therefore, that GHRH might be involved in the mediation of the sleep-promoting activity of ghrelin but not in ghrelin-induced feeding. In fact, feeding is attributed to ghrelin-induced stimulation of neuropeptide Y-containing neurons in the arcuate nucleus (9,43).

OCT elicits prompt suppression of GH secretion in rats (4). This effect is mediated in part via sst2 somatostatin receptors expressed by the pituitary somatotroph cells (42), and in part via sst2 receptors inhibiting GHRHergic neurons in the hypothalamus (24,59). OCT also elicits immediate inhibition of sleep followed by enhancements in EEG SWA during NREMS starting in hours 2 to 3 postinjection (4,16). The changes in GH secretion and sleep after OCT correlate in time with the variations in hypothalamic GHRH contents elicited by OCT (14). Although GHRH might be involved in the sleep response to OCT, somatostatin is a ubiquitous neurotransmitter in the CNS, and OCT may elicit a number of effects which inhibit sleep without directly interfering with GHRH. In fact, OCT induces drinking, vasopressin secretion and rises in blood pressure, which are attributed to a stimulation of intrahypothalamic angiotensin II release (17). Experiments in the lit/lit mouse demonstrate that the sleep response to OCT is absent in these mice with non-functional GHRH-Rs whereas OCT-induced drinking seems to persist. These results suggest that functional GHRH-Rs are necessary for the sleep effects of OCT, and that the OCT-induced angiotensinergic activity might not be dependent on GHRH action. That the sleep response to OCT is independent of angiotensin was previously reported; inhibition of angiotensin blocks the effects of OCT on drinking while not interfering with the sleep responses (4).
In conclusion, the results from the *lit/lit* mice suggest that the decrease in REMS is linked to GH deficiency, and that somatostatin and ghrelin may modulate NREMS via GHRH. The NREMS deficit in the *lit/lit* mice is consistent with the proposed importance of GHRH in the regulation of NREMS though other factors may also contribute to this alteration.
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REFERENCES


FIGURE LEGENDS

Fig. 1. *Lit/lit* mice sleep less than heterozygotic controls. Mean (± SE) hourly values of NREMS, REMS and the power of EEG slow wave activity (0.5-4 Hz) during NREMS (SWA) in heterozygous mice (open triangle, n=26), *lit/lit* mice (open circle, n=25), and *lit/lit* mice after 8-9 days of subcutaneous infusion of growth hormone (n=4: 11 µg/day, plus n=8: 24 µg/day; closed circle). Horizontal bar in the top marks the dark period of the day.

Fig. 2. Mean (± SE) hourly values of NREMS, REMS, and EEG slow wave activity during NREMS (SWA) on the baseline day (open symbols) and on the day of sleep deprivation (closed symbols) in heterozygous and *lit/lit* mice (n=13 in each group). Sleep deprivation was performed during the first 4 hours of the light period. Horizontal bars at the top mark the dark period. The % deviations from baseline values are depicted for SWA.

Fig. 3. Effects of intraperitoneal injection of octreotide (OCT, 200 µg/kg; n=11 heterozygous, and n=12 *lit/lit* mice), GHRH (5 µg/kg; n=14 heterozygous, and n=13 *lit/lit* mice), and ghrelin (400 µg/kg; n=11 heterozygous, and n=11 *lit/lit* mice) on NREMS. Changes in EEG SWA during NREMS are depicted for OCT as percentage difference between the vehicle day and the OCT day (columns). EEG SWA was not determined for hour 1 because the number of 10-s NREMS bouts was too low or NREMS did not occur after OCT in some mice. Closed symbols: injection of peptides; open symbols: injection of vehicle. The sleep effects of OCT were studied during the light period (injection at light onset) whereas the sleep effects of GHRH and ghrelin were studied during the dark period (injection at dark onset). Asterisks denote significant
differences in hour 1 between the peptide day and the vehicle day, and significant increments in SWA in hour 2 after OCT in the heterozygous mice.