Growth hormone-releasing hormone: cerebral cortical sleep-related EEG actions and expression

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Growth hormone-releasing hormone (GHRH) in non-rapid eye movement sleep (NREMS) regulation (reviewed in Ref. 19). Microinjection of GHRH into the anterior hypothalamus promotes NREMS, while injection of a GHRH antagonist peptide reduces duration of NREMS (38). Collectively, these and other data support the hypothesis that there are two parallel outputs of hypothalamic GHRHergic neurons, one stimulating pituitary GH and indirectly REMS, and the other directly regulating NREMS (reviewed in Ref. 2). The view that hypothalamic GHRHergic mechanisms are part of the NREMS regulatory network is consistent with the dominant paradigm in sleep research, suggesting that hypothalamic sleep regulatory networks impose NREMS on the brain (e.g., 29).

Previously, we (5, 26) and others (10, 33) described the presence of GHRH and GHRHR in the cortex. In spontaneous dwarf rats, their levels are different from controls, thereby suggesting that cortical levels of these molecules are regulated (26). However, it was not known whether cortical GHRH was active within the cortex or whether cortical GHRH, or its receptors, were equivalent to hypothalamic and pituitary forms. Herein, we demonstrate that GHRH applied to the cortex alters EEG delta power locally and that cortical GHRH does not restore REMS but not NREMS (20). Spontaneous dwarf rats, which have severe GH deficiency and high hypothalamic GHRH mRNA and GHRH release, display excess spontaneous NREMS (26). Hypothalamic GHRH mRNA and GHRH peptide content correlate with sleep propensity in normal and sleep-deprived rats (reviewed in Ref. 19). Microinjection of GHRH into the anterior hypothalamus promotes NREMS, while injection of a GHRH antagonist peptide reduces duration of NREMS (38). Collectively, these and other data support the hypothesis that there are two parallel outputs of hypothalamic GHRHergic neurons, one stimulating pituitary GH and indirectly REMS, and the other directly regulating NREMS (reviewed in Ref. 2). The view that hypothalamic GHRHergic mechanisms are part of the NREMS regulatory network is consistent with the dominant paradigm in sleep research, suggesting that hypothalamic sleep regulatory networks impose NREMS on the brain (e.g., 29).

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METHODS

Microinjection of GHRH Onto the Cortex and EEG Analyses

Animals and surgical procedures. Institutional guidelines for the care and use of research animals were followed, and the experimental protocols were approved by the Institutional Animal Care and Use Committee. Using ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia, we implanted male Sprague-Dawley rats (300–350 g) (Taconic Farms, Germantown, NY) with nuchal electromyographic (EMG) electrodes and cortical EEG electrodes over the somatosensory cortex (coordinates were 2.5 mm posterior and 5.5 mm lateral to the bregma) on both sides of the brain (36). Another EEG electrode, used as the common reference, was implanted 10 mm posterior from...
the bregma in the midline over the cerebellum. Rats were provided with guide cannulas for injection with their tips positioned under the EEG electrodes between the surface of the somatosensory cortex and the dura on each side of the brain. The guide cannulas and the screws were fixed to the skull with dental cement. After the surgeries, the animals were placed in individual sleep-recording cages in sound-attenuated, temperature-regulated environmental chambers at 24 ± 1°C ambient temperature on a 12:12-h light-dark cycle (light on at 0900) for habituation to the experimental conditions for at least 7 days. During this adaptation period, the animals were connected to the recording cables and handled daily to habituate them to the injection procedure. Water and food were available ad libitum during the experiments.

Verification of cannula placement. To verify the location of the microinjection cannulas, 2 μl of 20% lidocaine was injected on the surface of the cortex under ketamine-xylazine anesthesia. This procedure was carried out at least 1 wk before the animals were used in experiments. As we previously described, lidocaine decreases the EEG power in all frequency bands on the injected side of the brain (37). Only animals with correct placement of cannulas (decrease in EEG power on the injection side after lidocaine) were included in the data analysis.

Sleep-wake recordings. The digitized (128 Hz sampling rate) signals of the EEG and EMG were collected by computers. The EEG was filtered below 0.1 Hz and above 40 Hz. The states of vigilance were visually determined off-line in 10-s epochs by using the conventional criteria that we described previously (38). EMG activity served to aid in determining the vigilance state and was not analyzed further. The amount of time spent in each vigilance state was calculated for 3-h intervals during the recording period. EEG power values for the 0.5- to 4-Hz delta range during NREMS were integrated and used for characterizing NREMS intensity, also known as EEG slow-wave activity (SWA). On the baseline day, power density values in the delta range were averaged across the entire 23-h recording for each rat to obtain a reference value for that rat. Slow-wave activities during NREMS for each hour on the baseline day and the test days were expressed as a percentage of that reference value for each rat. Thus for the group, the average of all values shown in Figs. 1 and 2 for each side would be 100; as anticipated, daytime values are, in general, greater than night time values because rats sleep more during the day. The same data processing was done for both the injected side and the contralateral side. In addition, EEG power density values were calculated for each vigilance state from all artifact-free epochs by fast-Fourier transformation for consecutive 10-s epochs in the frequency range of 0.5–20.0 Hz in 0.5-Hz bands for each side of the brain under baseline and experimental conditions for the first 3-h time block of the recording period (Figs. 3 and 4). Values are means ± SE expressed as percentage of mean total power across the typical frequencies of the behavioral states.

Experimental Protocol

Experiment 1. Unilateral cortical administration of GHRH at light onset. Eleven rats were used for these experiments. The injections were performed 10 to 15 min before light onset. The injected volumes were 2 μl per injection site administered over 30 s. On the control day, the animals received 2 μl pyrogen-free isotonic NaCl. The experimental day occurred the day after control injections. They were injected with one of the following doses of GHRH (Sigma-Aldrich, St. Louis, Mo) onto the same side of the brain: 5 nmol (injection side right: n = 5; left n = 3), 0.5 nmol (injection side right: n = 6; left n = 3) and 0.05 nmol (injection side right: n = 5; left n = 3) dissolved in isotonic NaCl. Nine rats were injected with more than one dose of GHRH; the treatment days, which were always preceded by a control injection day, were separated by at least 3 days during which no treatment was given to animals. These rats were not selected based on previous responses to GHRH and when possible, the opposite side of the brain was injected. Immediately after injection, the animals were returned to their home cages. Sleep-wake activity was recorded for 23 h starting from the beginning of light onset (0900).

Experiment 2. Unilateral cortical administration of GHRH at dark onset. Ten rats were used for these experiments; none of these rats were used for light onset injections. The injections were performed 10 to 15 min before dark onset following the same protocol that we described above [5 nmol (injection side right: n = 5; left n = 4), 0.5 nmol (injection side right: n = 5; left n = 4) and 0.05 nmol (injection side right: n = 6; left n = 4)]. All 10 rats were used 2 or 3 times, and when possible, the opposite side of the brain was used.

Statistical Analysis

Two-way ANOVA for repeated measures was performed on sleep and power spectra data (factors: treatment and time effect or treatment and frequency effect, respectively). For SWA analysis, the hours during which a rat did not have at least 5 min NREMS were excluded. The exclusions resulted in occasional missing data points. Therefore, instead of repeated-measures ANOVA, two-way ANOVA was performed on SWA. Time spent in sleep and SWA data were analyzed in 3-h time blocks for the 23-h recording period between the baseline day and the experimental days in each group. Average power spectra values during wake, NREMS, and REMS were analyzed in the first 3-h time block after injections. When ANOVA indicated significant effects, post hoc comparisons were performed using the Student-Newman-Keuls test to identify which group and treatment differed from the other groups and treatments. The average episode duration and total number of vigilance state episodes between the baseline and experimental days were compared by paired Student’s t-test. An α-level of P < 0.05 was considered to be significant.

Expression of GHRH and GHRHR in the Cortex

Rat sample collection. Male Sprague-Dawley rats (250–350 g) adapted for at least 1 wk to a 12:12 h light-dark cycle (lights on at 0900) were used in time-of-day and sleep deprivation experiments. Rats were killed by decapitation every 4 h (1000, 1400, 1800, 2200, 0200, and 0600 h). Eight animals were killed at each time point, and the cerebral cortex was collected for protein analysis (Fig. 7). Eight additional control and eight sleep-deprived rats were killed at 1700 following sleep deprivation for 8 h after light onset; cortical tissue was analyzed (Fig. 8). Three rats were killed and the pyriform cortex, prefrontal cortex, somatosensory cortex and pituitary were collected for DNA sequence analysis and the parietal cortex for quantitative mRNA expression analysis (Fig. 6). All samples were quickly dissected and immediately placed in liquid nitrogen and stored at −80°C until further processing.

RNA Isolation. The RNA extraction, cDNA preparation, PCR amplification and gel electrophoresis were performed as described previously (5). Total RNA was extracted from different brain regions by the acid guanidinium-phenol-chloroform method using TRIzol reagent according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). At the end of the isolation procedure, the RNA pellets were suspended in DNase I buffer, which contained 1 U SUPERase-In and 2 U DNA-free (Ambion, Austin, TX) and incubated at 37°C for 90 min to degrade contaminating DNA. RNA concentration was estimated by UV absorbance at 260/280. The quality of the RNA was verified by 1% agarose gel electrophoresis.

cDNA synthesis. For PCR and real-time PCR analysis of the time-of-day samples collected every 4 h, cDNA was synthesized using Superscript III (Invitrogen). Two micrograms of total RNA were heated together with 0.5 μg oligo (dT)12–18 and 1 μl of 10 mM dNTPs at 65°C for 5 min, then chilled on ice. 0.1 M DTT, RNaseOUT (Invitrogen), 200 U of Superscript III, and 5X first-strand buffer were added, and the mixture was incubated at 55°C for 60 min. The reaction was stopped by incubating at 70°C for 15 min and then cooled to 4°C. The RNA template was degraded for 20 min at 37°C
with RNase H. Samples were diluted with sterile DNase-free water, aliquoted, and stored at −20°C.

**PCR of GHRH and GHRHR in brain samples.** cDNA was PCR amplified with primers to identify the presence of mRNA for GHRH and the three isoforms of the GHRHR in the pituitary, hypothalamus, and cortex. The PCR reaction used either HotStarTaq Master Mix (Qiagen, Valencia, CA) or Platinum Taq DNA Polymerase (Invitrogen). Primer sequences for GHRH were CTACGTGCTCTGGACATGC (1–20) and TTGCAGATGAGATGGGTCTTT (609–589), and primers for GHRHR and cyclophilin A were published (32). The PCR GHRH reaction used the following conditions: Hot-start: 15 min at 95°C before the three-step PCR, which consisted of 40 cycles of I) template denaturation at 95°C (15 s); 2) primer annealing at 58°C (15 s); and 3) product extension at 72°C (15 s). The final extension cycle was 10 min at 72°C. The GHRHR PCR used the following conditions: Initiation was 2 min at 94°C. The three-step PCR consisted of 35 cycles of I) denaturation at 94°C (30 s); 2) annealing at 57°C (30 s); and 3) extension at 72°C (60 s). The final extension cycle was at 72°C (5 min). PCR products were separated by 1 or 1.5% agarose gel electrophoresis and stained with ethidium bromide, and bands were visualized by UV transillumination.

**Real-time PCR measurement of GHRH and GHRHR in cortical samples.** Real-time PCR reactions were performed as previously described (32). Briefly, the PCR reaction mixture (25 μl) contained 5 μl diluted cDNA (100 ng total RNA equivalents for GHRHR and GHRH; 10 ng for cyclophilin A), 12.5 μl 2X PLATINUM Quantitative PCR SuperMix-UDG (Invitrogen), 0.25 μl of 1:1,000 dilutions of SYBR Green (Invitrogen) and fluorescein (Bio-Rad), and 0.5 μl of 10 μM primers for GHRH-R, GHRH, and cyclophilin A. PCR amplification conditions were the same as described previously (32). The binding of the PCR product with SYBR Green during the extension step of the PCR cycle emitted fluorescence at 495 nm, which was used to measure threshold cycles (Ct). Reactions were performed in triplicate, and Ct values were averaged. Gene expression was analyzed by the comparative Ct method using the formula: 2^−ΔΔCt, as described earlier (32). The fold-changes between the mRNA expression levels of the experimental and the control samples were calculated (32). The dissociation curves of used primer pairs showed a single peak, and PCR products had a single expected DNA band when run on an agarose gel (data not shown).

**Gel purification of GHRHR PCR product.** GHRHR PCR products were pooled to 200-μl total volume for each part of the brain and separated by electrophoresis on 1% agarose/bromide gels and visualized by UV light. The DNA bands were excised from the gels and purified by MinElute Gel Extraction Kit (Qiagen) according to manufacturer’s instructions. The excised DNA fragments were mixed with 3 volumes of buffer QG (wt/vol) and incubated at 50°C for 10 min. Following dissolution of the gel slice, 1 gel volume of isopropanol was added. This mixture was transferred to a MinElute column and centrifuged at 10,000 g for 1 min to bind DNA. Five-hundred microliters of QG buffer was added to the column, allowed to stand for 1 min, and washed with 750 μl PE buffer.

### Table 1. The amounts of wakefulness, non-rapid eye movement sleep, and rapid-eye movement sleep after saline and GHRH injections

<table>
<thead>
<tr>
<th></th>
<th>Dark-Onset Injections</th>
<th>Light-Onset Injections</th>
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<tbody>
<tr>
<td></td>
<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
</tr>
<tr>
<td>Amount of Wake</td>
<td>664.5±19.6</td>
<td>480.6±18.2</td>
</tr>
<tr>
<td>Amount of NREMS</td>
<td>592.9±18.4</td>
<td>208.1±14.5</td>
</tr>
<tr>
<td>Amount of REMS</td>
<td>122.6±5.0</td>
<td>31.4±4.5</td>
</tr>
<tr>
<td></td>
<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
</tr>
<tr>
<td>Amount of Wake</td>
<td>673.1±19.0</td>
<td>474.9±15.4</td>
</tr>
<tr>
<td>Amount of NREMS</td>
<td>581.0±16.9</td>
<td>211.5±13.4</td>
</tr>
<tr>
<td>Amount of REMS</td>
<td>125.9±4.52</td>
<td>33.5±3.5</td>
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<td></td>
<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
</tr>
<tr>
<td>Amount of Wake</td>
<td>670.2±26.5</td>
<td>482.6±23.8</td>
</tr>
<tr>
<td>Amount of NREMS</td>
<td>573.8±28.0</td>
<td>208.1±19.9</td>
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<tr>
<td>Amount of REMS</td>
<td>117.8±3.9</td>
<td>29.3±4.4</td>
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<td></td>
<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
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<tr>
<td>Amount of Wake</td>
<td>677.8±27.7</td>
<td>233.0±12.7</td>
</tr>
<tr>
<td>Amount of NREMS</td>
<td>596.9±28.4</td>
<td>400.4±15.2</td>
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<tr>
<td>Amount of REMS</td>
<td>103.5±9.2</td>
<td>84.8±10.0</td>
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<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
</tr>
<tr>
<td>Amount of Wake</td>
<td>698.3±16.7</td>
<td>240.2±19.5</td>
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<tr>
<td>Amount of NREMS</td>
<td>592.1±15.6</td>
<td>409.1±16.8</td>
</tr>
<tr>
<td>Amount of REMS</td>
<td>88.7±11.3</td>
<td>69.7±10.6</td>
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<tr>
<td></td>
<td>Saline, min/23 h</td>
<td>Saline, min/12 h</td>
</tr>
<tr>
<td>Amount of Wake</td>
<td>687.0±16.7</td>
<td>225.5±10.2</td>
</tr>
<tr>
<td>Amount of NREMS</td>
<td>589.5±10.9</td>
<td>420.0±12.1</td>
</tr>
<tr>
<td>Amount of REMS</td>
<td>102.7±13.5</td>
<td>73.8±8.3</td>
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Values on the control days are shown for the entire 23-h recording period and also separately for the first 12 h after saline injection and the following 11-h periods. NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep. Data are expressed as means ± SE.

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DNA was eluted with 10 µl of nuclease-free water, and the concentration was measured by UV absorbance at 260 nm.

Sequencing of GHRHR and GHRH from brain regions. GHRH PCR products were ligated into PCR 2.1 TOPO Vector with the TA Cloning Kit (Invitrogen), and bacteria were transformed with the plasmid. Bacteria were selected by PCR screening of boiled bacteria colonies for plasmids containing GHRH inserts using GHRH PCR primers. Positive colonies were grown overnight in Luri-Butani media containing ampicillin. Plasmids were purified using the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI). The GHRH insert was sequenced by BigDye Terminator Cycle Sequencing (Applied Biosystems, Foster City, CA).

Sixty micrograms of total protein was separated by electrophoresis through a denaturing 12% SDS polyacrylamide gel, and the proteins were transferred to a nitrocellulose membrane. Nonspecific protein binding to the membrane was blocked by incubation in Tween Tris-buffered saline (TTBS) buffer (0.01 M Tris, 0.15 M NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature. The membranes were then bathed in a 1:5,000 dilution of anti-GHRHR antibody (Bio-Synthesis, Lewisville, TX) or mouse anti-rat GAPDH polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% nonfat dry milk/TTBS overnight at 4°C. The anti-GHRHR antibody was developed by immunizing rabbits with a peptide sequence that matched the third extracellular loop of the protein (NH2-GCDAGLGIRPLE-OH) (26). After three washes in TTBS, the nitrocellulose membranes were incubated in either a 1:4,000 dilution of goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (GHRHR) or a 1:5,000 dilution of goat anti-mouse HRP conjugated secondary antibody (GAPDH) (DakoCytomation, Glostrup, Denmark). Immunoreactive protein was detected by the enhanced chemiluminescence detection reagent (ECL kit) according to manufacturer’s instructions (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and bands were visualized by exposure to autoradiographic film. Cruz Marker (Santa Cruz) molecular weight standards were used.
to identify the band sizes. The normalized values for each of the four gels (2 cortical samples/gel; n = 8) were averaged to yield one value for each of the 6 time points (Fig. 7). Data (Fig. 7) were subjected to one-way ANOVA. An alpha level of \( P < 0.05 \) was accepted as significant. Student’s \( t \)-test was used to compare the control and experimental values (Fig. 8).

RESULTS

Effects of GHRH on Cortical EEG

At dark onset, none of the doses of GHRH microinjected unilaterally onto the cortex altered the duration of NREMS or REMS, nor were the distributions of these states across the recording period changed (Table 1). Thus, greater duration of NREMS and REMS was observed during daylight hours than during night time hours after all of the doses (data not shown). Similarly, after light-onset injections of GHRH, none of the doses altered the duration or distribution of NREMS. In contrast, the two lower doses of GHRH, 0.05 and 0.5 nmol, injected at the onset of daylight, enhanced REMS for about 6 h. The amount of time (in minutes) in REMS during the first 6 h of daylight was 47.5 \pm 5.6 vs. 30.0 \pm 5.3 for 0.05 nmol vs. saline, respectively, and 43.8 \pm 1.9 vs. 30.6 \pm 5.6 for 0.5 mol vs. saline, respectively. The total number of REMS episodes for the first 6 h was 17.3 \pm 2.4 after saline vs. 21.3 \pm 2.1 after 0.05 nmol GHRH (\( P > 0.05 \), Student’s \( t \)-test). The average duration of REMS episodes was 94.1 \pm 10.4 s after saline vs. 127.2 \pm 2.0 s after 0.05 nmol GHRH (\( P < 0.05 \), Student’s \( t \)-test). The total number of REMS episodes for the first 6 h was 15.2 \pm 2.4 after saline vs. 20.7 \pm 1.0 after 0.5 nmol GHRH (\( P < 0.05 \), Student’s \( t \)-test). The highest dose of GHRH tested (5 nmol) did not affect duration of REMS or its distribution across the recording period (data not shown). After the cortical injections of GHRH, no behavioral abnormalities were observed; that is, the animals continued to cycle through bouts of wakefulness and sleep.

Unilateral injection of the 5-nmol dose at dark onset enhanced EEG slow-wave power compared with values obtained on the baseline day from the same side of the cortex during the first 3 h postinjection (Fig. 1) [ANOVA, treatment effect \( F(1,126) = 9.3, P < 0.05 \)]. In contrast, the lowest dose tested, 0.05 nmol, reduced EEG delta wave power ipsilaterally during this period compared with the baseline day values [ANOVA, treatment effect \( F(1,140) = 13.9, P < 0.05 \)]. Similar results were obtained after daylight-onset injections. Thus, the higher dose enhanced EEG delta power during the immediate postinjection hours on the side ipsilateral to the injection [ANOVA, treatment effect \( F(1,112) = 4.6, P < 0.05 \)], while the lowest dose decreased EEG delta power for about 6 h compared with baseline values (Fig. 2) [ANOVA, treatment effect, \( F(1,141) = 4.3, P < 0.05 \)]. The 0.5 nmol dose did not produce a significant effect whether given at daylight or dark onset.

If comparisons of the effects of GHRH were made between the side ipsilateral to the injection to the contralateral side, frequency- and state-dependent changes in EEG power were observed. The 5.0 nmol dose enhanced EEG delta power during both wake [ANOVA, treatment \( \times \) frequency interaction
$F(39,312) = 2.5, P < 0.05$] and NREMS [ANOVA, treatment × frequency interaction $F(39,312) = 3.1, P < 0.05$] episodes on the ipsilateral side compared with the contralateral side (Fig. 3) when injected at dark onset. No differences in power were observed during REMS at this dose. The 0.5-nmol dose only enhanced EEG delta power during NREMS [ANOVA, treatment × frequency interaction, $F(39,312) = 7.3, P < 0.05$] and was without effect during waking or during REMS. In contrast, the lowest dose inhibited EEG delta power during NREMS [ANOVA, treatment × frequency interaction $F(39,273) = 1.6, P < 0.05$] but was without effect during waking and REMS (Fig. 3). Similar results were obtained after light-onset injections. Thus, the high dose of GHRH enhanced EEG delta power [ANOVA, treatment × frequency interaction, $F(39,273) = 2.8, P < 0.05$], while the low dose reduced it during NREMS, but this latter effect was not significant (Fig. 4). The middle dose of GHRH was without effect on EEG power during NREMS. None of the doses altered EEG power in any frequency during waking or REMS after light-onset injections (Fig. 4).

**GHRH and GHRHR Cortical Expression**

**GHRH.** The sequences of GHRH mRNA extracted from the pituitary, hypothalamus, and cortex were identical (data not shown); the base sequence corresponding to the 44 amino acid mature GHRH was found. Upon gel electrophoresis, GHRH mRNA from all sources had the same mobility (Fig. 5). Similarly, the GHRHR forms in the pyriform cortex, prefrontal cortex, and somatosensory cortex were similar to those found in the pituitary. Two GHRHR mRNA forms were found (Fig. 6), and their mRNA sequences were identical in each tissue examined. By sequence analysis and a National Institutes of Health Blast search, we identified the 560-bp form as the short GHRHR isoform (NM-1629 bp) and the 429-bp beta GHRHR isoform (NM-1320 bp) was also present. The abundance of the 560-bp isoform was greater than the 429-bp form in the pituitary, although in the cortical samples, the two isoforms were roughly equal (Fig. 6).

Previously, we showed that there was not a pronounced daily rhythm in cortical GHRH mRNA (5). Preliminary data from similar samples indicated that GHRHR mRNA also did not vary across the day (data not shown). Consistent with preliminary RNA data, GHRHR protein expression in the cortex did not vary across the day by Western blot analysis (Fig. 7). In contrast to the lack of daily rhythms, sleep deprivation enhanced cortical GHRH mRNA, although GHRHR mRNA was not affected by sleep loss (Fig. 8).
DISCUSSION

The major findings presented herein are that GHRH and GHRHR are present in the rat cerebral cortex and that the GHRH/GHRHR system contributes to EEG delta wave activity. Previously, others had demonstrated the presence of GHRH/GHRHR and/or their mRNAs in cortical samples both in vivo and in vitro, although those studies did not fully characterize the substances or address their regulation in the cortex (9, 10, 12, 32, 34). We show here that the cortical forms of the substances are identical to their hypothalamic and pituitary forms, although the ratio of the low to high molecular weight forms of GHRHR found in the cortex was different than that found in the pituitary. Previously, we had demonstrated that the cortical expression of GHRH and GHRHR in spontaneous dwarf rats, which have lower circulating GH, was different from control rats with normal GH plasma levels (26). In that study, both GHRH protein and the number of GHRH-immunopositive perikarya were higher in the dwarf rats, suggesting some degree of expression regulation by GH analogous to that found in the somatotropic axis. Further, in spontaneous dwarf rats, cortical GHRHR mRNA and the number of cortical GHRHR-immunopositive cells were decreased compared with controls. This could reflect down-regulation of GHRHR by an increased GHRH cortical tone (4). Regardless, current data clearly show that the forms of GHRH/GHRHR found in the cortex are the same as those found elsewhere in brain.

We also showed that cortical GHRHRs are functional; thus unilateral application of GHRH to the surface of the cortex ipsilaterally enhanced EEG delta wave power at high doses and decreased EEG delta wave power at the low dose tested. After the lower doses, the reduction in EEG SWA occurred on a higher background of delta power, and this warrants caution with the interpretation of the data. However, the biphasic response seemed to be independent of the light-dark cycle, since it was observed after both light- and dark-onset injec-

Fig. 6. A: electrophoresis of RT-PCR-assisted amplification of GHRH receptor (GHRHR) mRNA from four brain areas of normal rats. An ethidium bromide-stained agarose gel (1%) is shown; the top band is 560 bp and the bottom band is 429 bp. Lane 1: pituitary (Pit), lane 2: piriform cortex (PC), lane 3: prefrontal cortex (PFC), lane 4: somatosensory (SSctx), and lane 5: control. B: sequence analyses identified 560-bp (top) and 429-bp (bottom) bands-recoding (L01407 1629 bp) and (AF122055 1320 bp) GHRHR isoforms, respectively, by National Institutes of Health sequence homology software BLAST. The 429-bp GHRHR lost 131 bp between AAGG and TCCC compared with the 560-bp sequence.

Fig. 7. Time-of-day variation in the relative amounts of GHRHR and GAPDH protein in the rat cerebral cortex by immunoblot analysis. A: lanes illustrate the expression of GHRHR (top) and GAPDH (bottom) protein at six time points: 1000, 1400, 1800, 2200, 0200, and 0600, respectively. Samples from 2 rats are shown for each time point. B: data from four separate gels were averaged; each gel was as in A with two rats/time point. Means ± SE (n = 8) is shown for each time point. The horizontal black bar indicates the dark period.

Fig. 8. Effects of 8 h of sleep deprivation on GHRH and GHRHR mRNA expression in the rat cerebral cortex. Amounts of GHRH and GHRHR mRNA in experimental (black bars) and time-matched control (gray bars) samples are expressed. SD, sleep deprivation. Data are presented as means ± SE of values obtained. *Significant difference from control and experimental day (P < 0.05, Student’s t-test).
tions. We currently do not understand why the direction of the effect of GHRH on EEG delta power is dependent on dose, although GHRH can down-regulate its receptor (4).

Microinjection of GHRH into the preoptic anterior hypothalamus enhances EEG delta power and duration of NREMS (38). This finding was interpreted as being a manifestation of GHRH acting on those sleep mechanisms engaged during sleep loss because during the deep NREMS occurring after sleep deprivation, EEG delta activity is enhanced (25). However, because we now show that cortical application of GHRH can either increase or decrease EEG delta power, the relationship of EEG delta waves to NREMS needs to be revisited. These two sleep phenotypes, duration of NREMS and EEG delta power, previously were shown to be, in part, regulated independently. Thus, for example, in neonates and aging individuals, NREMS often is unaccompanied by EEG delta waves and substances that enhance both duration of NREMS and EEG delta power in adults only enhance duration of NREMS in neonates (7). Brain lesions can cause a decrease in EEG delta power without long-term substantial changes in duration of NREMS (14, 30). Systemic atropine can enhance EEG delta waves, without affecting state (31). Regardless of such evidence, EEG delta power is a remarkably good parameter to use for process S in the two-process model of sleep (1). Further, in many normal and experimental situations, EEG delta power is correlated with the intensity of NREMS (e.g., 24). How cortical GHRH/GHRHR is involved in these observations is in need of clarification.

Certain sleep regulatory substances such as IL-1 beta and TNF, but not others, such as prostaglandin D2 or an adenosine agonist, also enhance EE delta power unilaterally if applied to the surface of the cerebral cortex (36, 37). Although both of these substances were shown only to enhance EEG delta power after local application to the cortex, in the case of IL-1, the middle dose enhanced EEG delta power more than the highest dose tested. This suggests the possibility that its actions are also biphasic. In cultured hypothalamic neurons, there are GABAergic cells that are responsive to both IL-1 and GHRH, as determined by intracellular Ca++ responses (8). In preliminary data from our laboratory (17), some cultured cortical neurons also have enhanced intracellular Ca++ in response to GHRH application; we are in the process of determining whether they are also responsive to IL-1, as are hypothalamic neurons. It is tempting to speculate that such GHRH- and IL-1-responsive cortical neurons are key to the regulation of cortical EEG delta power. Regardless of such speculation, it is currently unknown whether the amounts of GHRH/GHRHR normally present in the cortex increase or decrease EEG delta power.

A new theory of brain organization of sleep and sleep regulation posits that NREMS is a fundamental property of neuronal networks (15, 16). It is proposed that sleep is initiated within neuronal assemblies (e.g., cortical columns) as a function of prior cellular activity, that is, that neuronal activity enhances production and release of sleep regulatory substances such as GHRH that in turn act locally to affect cortical column state. Indeed, Rector et al. (27) demonstrated that cortical columns oscillate between functional sleep- and wake-like states as defined by amplitudes of surface-evoked potentials. The probability of sleeplike state occurrence increases the longer the cortical column is in the wake-like state. Furthermore, in the cortical column a sleep-like state most often occurs during whole animal sleep. In a learning paradigm that uses facial whisker stimulation and is dependent on a single cortical column, in the response error rate is higher if the column is in the sleeplike state (35). Cortical microinjection of TNF, a well-characterized sleep regulatory substance, locally enhances EEG delta power during NREMS, suggesting greater localized NREMS intensity (37) and increases the probability of a cortical column entering the sleeplike functional state (6). Indeed, extensive whisker stimulation increases the probability of its corresponding somatosensory cortical column entering the sleeplike state and enhances TNF expression in the activated column (11). TNF is one of several sleep regulatory substances, and it has been linked to GHRH in that TNF alters pituitary release of GH via a hypothalamic mechanism (28). How GHRH may interact with either TNF or IL-1 in cortical tissues to affect EEG delta power or sleep at the neuronal assembly level is under investigation.

In summary, we showed that two elements of the somatotropic axis, GHRH and its receptor, are present in the cortex and seem to have a functional role in EEG delta wave activity that is state dependent. Data are consistent with the notion that sleep is a local property of cerebral cortical neuronal assemblies.

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