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Role of GHRH in Sleep and Growth Impairments Induced by Upper Airway Obstruction in Rats

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On-line Methods

The study was approved by the Ben-Gurion University of the Negev Animal Use and Care Committee and complied with the American Physiological Society Guidelines. **Surgery**: The technique used for sham surgery and to induce upper airway obstruction (UAO) in 22-day-old pre-puberty Sprague-Dawley male rats was as previously described. (E1–E4) A midline ventral cervical incision was made. The trachea was exposed and dissected so as not to damage adjacent structures. A circumferential silicon band 0.5 cm long was placed around the trachea. A suture was looped around the band and tightened, thus constricting the trachea so as to increase inspiratory esophageal pressure swings two–three-fold. Following a nine-day recovery period, a telemetric transmitter (TL11M2-F20-EET, Data Sciences International, DSI, St. Paul, MN, USA) was implanted (under sterile conditions), enabling recording of electroencephalography (EEG), dorsal neck electromyography (EMG) and body temperature. Leads from the electrodes for EEG recording were placed over the frontal (1.1 mm anterior and 1.1 mm lateral to the bregma) and parietal (3 mm posterior and 1.5 mm lateral to the bregma) cortices. EEG electrodes were anchored to the skull with dental cement. (E5,E6) All surgical procedures were performed under tribromoethanol (200 mg/kg ip) anesthesia. Following telemetric surgery, prophylactic enrofloxacin 5 mg/mL (s.c.) and water containing ibuprofen (0.1 mg/mL) were given for three days. (E6–E8) Animals were given food and water *ad libitum.*

Experimental schedule: UAO or sham surgery was performed on 22-day-old rats (day=0). Animals were returned to their home cages (23±1.0ºC) and allowed to recover for 9 days. They were kept in a 12:12 h light:dark cycle with light onset at

09:00, >500 lux. Following the nine-day recovery period, a telemetric transmitter was implanted for recording sleep and data were collected on day 15 continuously for 24 hrs. Longitudinal growth was measured on days 0 and 14 post-surgery. Respiratory parameters and arterial blood gases were measured 16 days post-surgery immediately before animal death. Determination of hypothalamic growth hormone receptor, growth hormone releasing hormone, growth hormone releasing hormone receptor, orexin mRNA and protein, serum growth hormone and insulin-like growth factor-1 levels was performed on separate series of animals 16 days post-surgery. The rats were killed by guillotine between 1 and 2 hours after light onset 16 days post-surgery. Serum and hypothalamus were collected within 1 minute after death.

Ritanserin study: Animals were injected i.p. with ritanserin or vehicle (4% methyl alcohol in saline) at lights on for 8 consecutive days. (E3) Four groups were used: 1) UAO + ritanserin, 2) UAO + vehicle, 3) control + ritanserin, 4) control + vehicle. On day 8, animals were sacrificed 1 to 2 hrs after light onset and the hypothalamus and serum were collected.

Sleep-wake recording and scoring

The signals were processed by a DataSciences analog converter and routed to a computer. The digitized signals (256 Hz sampling rate) were collected by computers; EEG and EMG signals were filtered at 0.1 and 40 Hz and 10 and 300 Hz, respectively. EMG and body movement activities aided in determination of the vigilance state of the animals. The vigilance states were scored using software (DSI NeuroScore v. 2.1 software) and were edited visually for 10 sec epochs, on the basis of the predominant state within the epoch, as follows (E9,E10): 1) slow wave sleep

(SWS); 2) paradoxical sleep (PS – equivalent to REM in humans); and 3) wake (W). The durations of each vigilance state (excluding artifacts) were calculated in 1-h time blocks as previously described (E5,E9–E11). Light slow wave sleep (SWS-1) was defined as high-voltage slow cortical waves (0.5–4 Hz) interrupted by low-voltage fast EEG activity (spindles, 6–15 Hz) accompanied by reduced EMG and motor activity. Deep slow wave sleep (SWS-2) was defined as continuous (>80% epoch) high-amplitude slow cortical waves (0.5–4 Hz) with reduced EMG and motor activity. Total SWS was defined as the sum of SWS-1 and SWS-2.

Power density was calculated during the first 3 hours of light on for 10 sec epochs in the frequency range 0.5–30 Hz (Hamming window), frequency resolution 0.5 Hz. Power densities were averaged separately for each vigilance state. (E12) The power density values for the 0.5–4.0 Hz (delta) frequency range were integrated and used as an index of EEG slow wave activity (SWA) during non-rapid-eye-movement sleep to characterize sleep intensity in each recording hour. (E11)

Longitudinal growth was measured before and 14 days after UAO or sham surgery by measuring tibial and tail lengths as previously described. (E1–E3,E13)

Respiratory parameters: Inspiratory swings in esophageal pressure and respiratory rate were measured 16 days post-surgery immediately before killing the animals. (E1–E3) At sacrifice (between 10:00 and 11:00), tracheal resistance was determined immediately after animal death as the slope of flow-pressure curves in the linear portion of the curve (0.1–0.3 L/min) (E3,E4) in 7 sham controls and 7 UAO rats. The serum was separated for later measurements of growth hormone and insulin-like growth factor-1 by specific ELISA kits (DSL, Webster, TX, USA).

Arterial blood gases: In a subset of 7 sham and 7 UAO animals, arterial blood gases (pH, PCO_2, PO_2 and HCO_3^-) and hemoglobin concentration were determined at one time interval 16 days after surgery. (E2,E3)

Food intake (E3,E4): In a subset of 8 sham controls and 9 UAO animals, food intake was determined on days 12 and 13 post-surgery. Animals were housed individually in cages and given 30 g/day of standard rodent chow (Harlan, Jerusalem, Israel). Food placed into the feeder at the beginning and any left over at the end of each 24-hr period was weighed. Any visible food in the cage was scavenged and included in the measurements. The standard rodent chow included protein (21%), fat (4%), carbohydrate (53.5%), moisture (13%), energy 3.95 (KCal/kg). Daily food intake is expressed as grams of food/kg of body weight.

Determination of mRNA expression level: To determine growth hormone releasing hormone mRNA expression level, a subset of 16 sham and 16 UAO rats was used. The hypothalamus/optic region (landmarks of frontal edge of the optic chiasma, lateral sulci and mammillary bodies, at a depth of 2 mm) was harvested and frozen as previously described. (E11,E14) Due to low level of hypothalamus mRNA level tissue from 2 animals were combined to one measurement. Total RNA was extracted from the hypothalamus using the PerfectPure RNA Tissue kit (Gentra Systems, Minneapolis, MN, USA). RNA was treated for DNA contamination using DNase solution (Gentra systems). RNA concentration was quantified by absorbance at 260 nm. cDNAs were synthesized from 1 μg of total RNA using high-capacity cDNA reverse transcription kit containing RT random primers, RNase inhibitor, Multiscribe Reverse Transcriptase, dNTPs mix and RT buffer (Applied Biosystems, Foster City,

CA, USA). The reaction was incubated at 25ºC for 10 min; 37ºC for 2 h; 85ºC for 5 sec. Quantitative real time PCR (qPCR) assays were carried out for growth hormone releasing hormone (GHRH) and β-actin mRNAs with the following primers (Sigma-Aldrich, Rehovot, Israel):

Growth hormone receptor Sense: 5'-ATCTTTGGCGGGTGTTCTTA-3'.

Growth hormone receptor Anti-sense: 5'-TAGCTGGTGTAGCCCCACTT-3'.

GHRH Sense: ACTCTGGGTGTTCTTTGTGC.

GHRH Anti-sense: CCCTTGCTGCCTGTTCATGAT.

Orexin Sense: 5′-TAGAGCCATATCCCTGCCC.

Orexin Anti-sense: 5′-GAGGAGAGGGGAAAGTTAGG.

β-actin Sense: GGTCTCAAACATGATCTGGG.

β-actin Anti-sense: GGGTCAGAAGAATTCCTATG.

Primer optimized concentrations were chosen according to primer optimized protocol (Applied Biosystems, Foster City, CA, USA). Real time PCR reactions were performed with power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7300 Sequence detection System (Applied Biosystems). Each sample was analyzed in triplicate (final reaction volume 20 μL) in 96-well Micro Optical plates (Applied Biosystems), each sample representing an individual assay. For each sample, 200 ng of cDNA was added to power SYBR green PCR master mix containing Rox (Applied Biosystems, Foster City, CA, USA) and 500nM primers. The PCR protocol was: 50ºC for 2 min; 95ºC for 10 min; and 40 cycles of 95ºC for 15 s followed by 60ºC for 1 min. The specificity of the reaction is given by the detection of the melting temperatures (Tms) of the amplification products immediately after the last reaction cycle. The target gene expression value

was calculated by the ΔΔct method after normalization with a housekeeping gene (β-actin).

Immunoassays: For determination of hypothalamic growth hormone releasing hormone content, a subset of 10 sham and 12 UAO rats was used. The rats were killed by guillotine between 1 and 2 hours after light onset and hypothalamus and serum were collected (see above). Blood and tissue samples were collected and frozen at -80°C until analysis. Serum IGF-I concentrations were measured using an ELISA kit (DSL-10-29200, Diagnostic Systems Laboratories, Inc., Webster, TX, USA). Serum growth hormone concentrations were measured using an ELISA kit (DSL-10-72100, Diagnostic Systems Laboratories). Hypothalamic growth hormone releasing hormone content was determined by specific ELISA kit for rats (E0438Ra, USCN Life Science Inc., Wuhan, China) following the manufacturer's instructions.

Online Results

As expected, immediately following UAO, inspiratory swings in esophageal pressure increased by 254%, respiratory rate decreased by 43% and tracheal resistance increased by 46% (Table 1E). No signs of gasping were observed among UAO animals. Arterial blood gases and hemoglobin were within normal range and unchanged in both groups (Table 1E).

Somatic growth: Both UAO and control groups had similar baseline body weight and tibial and tail lengths (p=0.28, 0.83 and 0.84, respectively; Table 2E). Differences between the growth curves of UAO and sham controls are significant at all time

intervals after surgery (p=0.002). Tibia and tail lengths were significantly less in UAO animals compared to controls (Table 2E).

Electroencephalogram power density was analyzed separately for each vigilance state during the first 3 hrs of the light period. When awake, the UAO group exhibited decreased electroencephalogram power in the frequency range of 0.5–2.5 Hz (Figure 1E). During SWS, the power density of UAO rats decreased by 40% at 1.5 Hz compared with controls (p<0.001, Figure 1E). No significant difference was noted in the power spectra density of PS during the first 3 hrs of light period.

Endocrine analysis: The hypothalamic growth hormone receptor mRNA (Figure 2E-A) and protein levels (Figure 2E-B) decreased in the UAO group by 38% (p=0.001) and 66% (p=0.001), respectively (10 animals were used for each group).

Orexin: Hypothalamic orexin mRNA (Figure 3E) increased in UAO by 73% (p<0.01).

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Table 1E. Respiratory changes 14 days post-surgery.

Variable	Control (n=8)	Airway loading (n=8)	*p* value
PO_2 (mmHg)	94.2± 10.1	95.9± 14.5	0. 8
PCO_2 (mmHg)	37.4±3.3	41.1± 9.3	0.4
pH (units)	7.35±0.03	7.34±0.07	0.8
HCO_3^- (mEq/L)	21.7±0.7	23.9±2.6	0.1
Hemoglobin (g/dL)	11.7±0.7	12.2±0.7	0.2
ΔPes (cm H_2O)	-8.1±3.2	-20.6±10.7	0.033
Respiratory rate (breaths/min)	126.0±12.0	71.6±15.2	<0.001
Tracheal resistance (mmHg/L/min)	3.9±1.4	5.7±1.4	0.035

HCO_3 = calculated arterial bicarbonate; PCO_2 = arterial CO_2 pressure; Pes = inspiratory swings in esophageal pressure; pH = arterial pH; PO_2 = arterial O_2 pressure. Values are mean±SD.

Table 2E: Somatic growth parameters.

	Sham Control (n=11)			UAO (n=11)		
	Day 0	Day 14	Gain	Day 0	Day 14	Gain
Body weight (gr)	51.8±1.7	148.6±5.3	98.0±4.3	52.5±1.7	112.5±10.6#	60.3±10.2 +
Tail length (mm)	101.6±6.6	169.4± 10.9	67.8±1.0	98.5±4.9	154.4±9.2#	55.8±11.2+
Tibial length (mm)	30.4±1.7	42.1±1.0	11.7±1.7	30.3±1.3	40.2±2.3#	9.9±2.6

Base line measurement immediately before upper airway obstruction (UAO) surgery (day 0). Body weight, tail and tibial gains were performed on n=11 control and n=11 upper airway obstruction (UAO) animals. # $p<0.01$ comparing day 0 with day 15 post-surgery. + $p<0.02$ comparing control gain with UAO gain. Values are mean (SD).

Figure legends

Figure 1E: Electroencephalogram power density in wake (W), slow wave sleep (SWS) and paradoxical sleep (PS) during the first 3 hrs of light for control (open symbols) and UAO (closed symbols). Each point represents mean values of 0.5 Hz bins in a 3-hr recording period. The curves connect absolute power density values. UAO decreased electroencephalogram power in the frequency range of 0.5–2.5 Hz in W. In SWS, UAO decreased electroencephalogram power in frequency range <5 Hz. # Indicates statistically significant difference between the groups, $p<0.0001$. Values are mean ± SEM.

Figure 2E: (A) Hypothalamus growth hormone receptor (GHR) relative mRNA level determined by real time PCR. (B) Top – representative Western immunoblot analysis of hypothalamus GHR and β actin from two control and two UAO animals. Bottom – densitometric analysis of GHR protein. # $p=0.001$.

Figure 3E: Hypothalamus orexin relative mRNA level determined by real time PCR. # $p<0.01$.

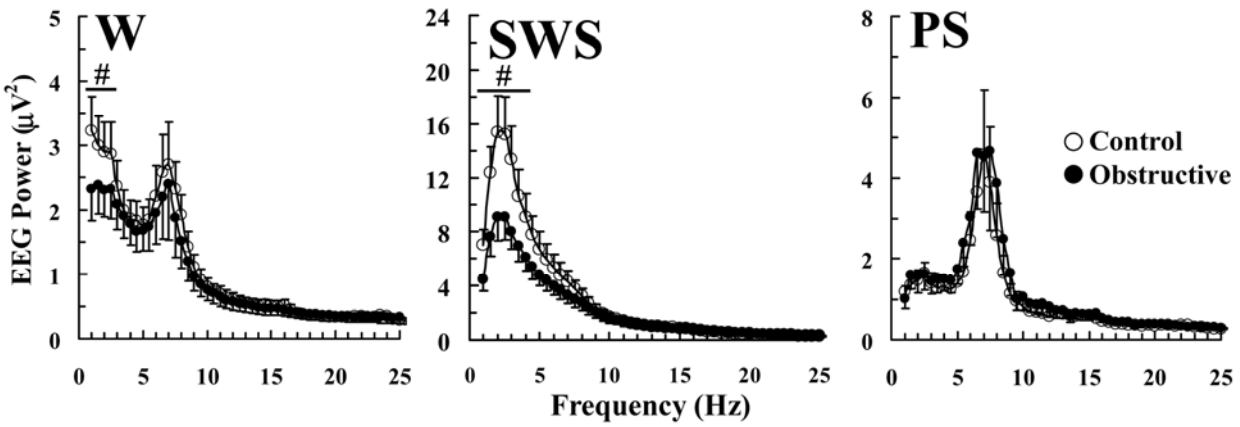

Figure 1E

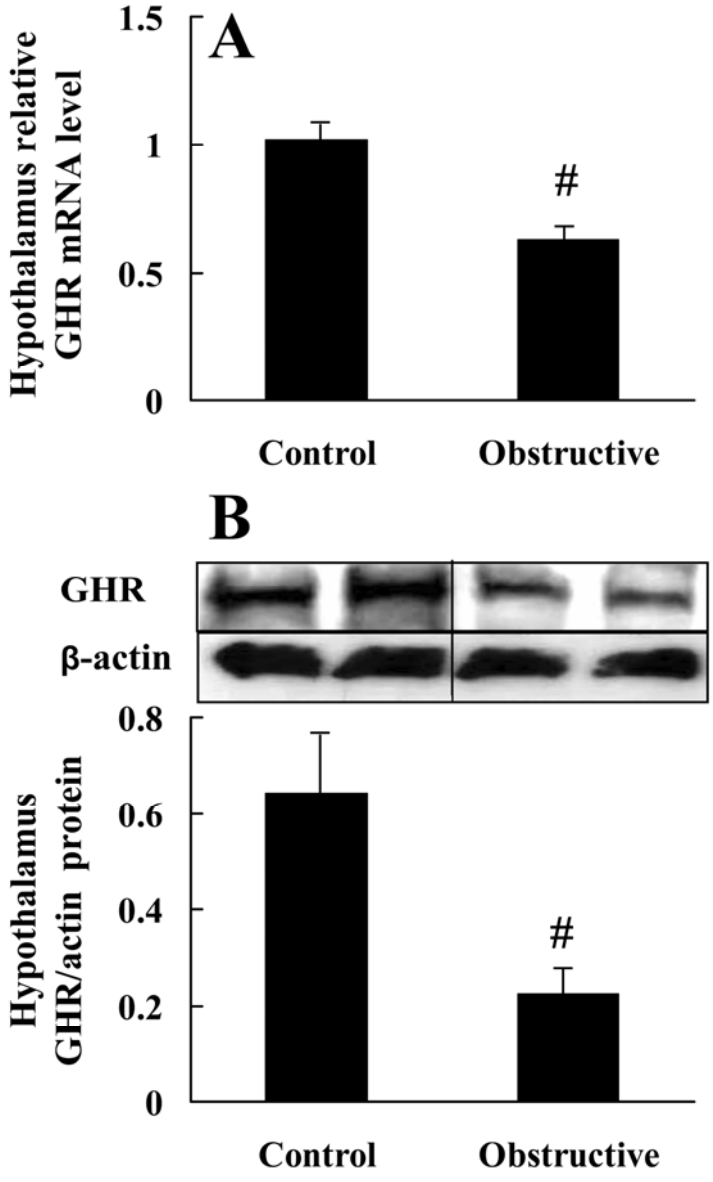
A
Hypothalamus relative GHR mRNA level
1.5
1
0.5
0
#
Control
Obstructive
B
GHR
β-actin
Hypothalamus GHR/actin protein
0.8
0.6
0.4
0.2
0
#
Control
Obstructive

Figure 2E

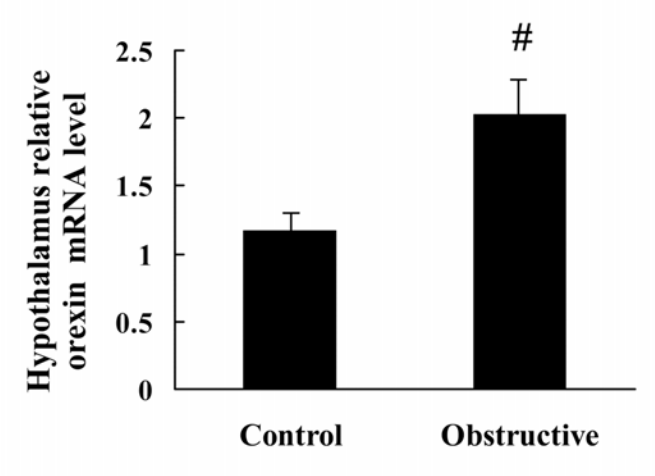
Hypothalamus relative orexin mRNA level
2.5
2
1.5
1
0.5
0
#
Control
Obstructive

Figure 3E