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Role of growth hormone-releasing hormone in sleep and growth impairments induced by upper airway obstruction in rats

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ABSTRACT: Upper airway obstruction (UAO) can lead to abnormal growth hormone (GH) homeostasis and growth retardation but the mechanisms are unclear. We explored the effect of UAO on hypothalamic GH-releasing hormone (GHRH), which has a role in both sleep and GH regulation.

The tracheae of 22-day-old rats were narrowed; UAO and sham-operated animals were sacrificed 16 days post-surgery. To stimulate slow-wave sleep (SWS) and GH secretion, rats were treated with ritanserin (5-HT_2 receptor antagonist). Sleep was measured with a telemetric system. Hypothalamic GHRH, hypothalamic GHRH receptor (GHRHR) and GH receptor, and orexin were analysed using ELISA, real-time PCR and Western blot.

UAO decreased hypothalamic GHRH, GHRHR and GH receptor levels, while orexin mRNA increased ($p<0.01$). In UAO rats, the duration of wakefulness was elevated and the duration of SWS, paradoxical sleep and slow-wave activity was reduced ($p<0.001$). Ritanserin alleviated these effects, *i.e.* normalised hypothalamic GHRH content, decreased wake duration, increased duration and depth of SWS, and attenuated growth impairment ($p<0.001$).

Here, we present evidence that growth retardation in UAO is associated with a reduction in hypothalamic GHRH content. Our findings show that abnormalities in the GHRH/GH axis underlie both growth retardation and SWS-disorder UAO.

KEYWORDS: Growth hormone axis, growth retardation, rat, sleep, upper airway loading

Children with upper airway obstruction (UAO) usually suffer from sleep-disordered breathing and growth retardation [1–4]. We previously hypothesised that the impairment of growth is related to reduced growth hormone (GH) release from the pituitary gland, which normally requires undisturbed slow-wave sleep (SWS) [1]. The release of GH is greatly enhanced during sleep, especially early in the night; this is associated with the appearance of delta waves on electroencephalography, which are characteristic of SWS [5], and increased release of GH-releasing hormone (GHRH) in the hypothalamus [6]. The mutual relationships between sleep and GHRH are complex. Central or systemic administration of exogenous GHRH enhances non-rapid eye movement (NREM) sleep, while inhibition of endogenous GHRH secretion suppresses sleep duration and depth [7–9]. Genetic mutations associated with GHRH deficiency lead to reduction of both depth and duration of NREM sleep and to a decrease in GH secretion, resulting in dwarfism [9].

The possibility that GHRH may be affected in children with sleep-disordered breathing emerges from studies showing that both slow-wave activity, a quantitative measure for NREM sleep depth [10], and GH axis are suppressed [1]. Moreover, all these sleep and hormonal disturbances, as well as growth retardation, are reversed following surgical removal of the UAO [1, 2, 10]. In adult humans, GH and slow-wave activity are normalised following treatment of sleep-disordered breathing with continuous positive airway pressure [11, 12].

Ritanserin, a selective 5-HT_2 receptor antagonist, increases endogenous GH release and NREM sleep [13]. The strong association between sleep and GH release raises the possibility that pharmacological agents that increase NREM sleep can also stimulate GH secretion [13].

We have used chronic UAO in juvenile rats as a model bearing some features of human sleep-disordered breathing and subglottic stenosis [14, 15]. In this model, we have shown that UAO

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causes growth retardation, which is related to an impairment of the global and the local GH/insulin-like growth factor (IGF)-1 axis. In parallel, UAO also fragments NREM sleep [16].

The mechanisms linking UAO with neuroendocrine disturbances, which cause growth retardation and sleep abnormalities, are unknown. In this study, we hypothesised that UAO affects sleep and growth by causing a decrease in hypothalamic GHRH levels. We found that UAO indeed causes depletion of hypothalamic GHRH. We also provide evidence that pharmacological blockade of 5-HT_2 receptors could prevent the UAO-induced depletion of the hypothalamic GHRH content, preserve sleep and attenuate growth retardation.

METHODS

Further methods used can be found in the online supplementary material.

Surgery

Tracheal narrowing surgery (anaesthesia 200 mg·kg^{-1} intraperitoneal tribromoethanol) was used to induce UAO in 22-day-old male Sprague-Dawley rats [14, 15]. Controls underwent surgery with no tracheal narrowing. Animals were returned to their cages and kept in 12/12-h light/dark cycle, with lights on at 09:00 h. On day 9, telemetric transmitters for sleep recording were implanted (see later). Food and water were provided *ad libitum*.

The study was approved by the Ben-Gurion University of the Negev Animal Use and Care Committee (Beer-Sheva, Israel) and complied with the American Physiological Society Guidelines.

Experimental schedule

Sleep was recorded on day 15 (baseline) and day 16 following acute administration of high-dose ritanserin (Sigma-Aldrich Ltd, Rehovot, Israel), a 5-HT_2 receptor antagonist, at 09:00 h. In a separate series of animals, hypothalamic GHRH, GH receptor, GHRH receptor (GHRHR), orexin mRNA and proteins, serum GH and IGF-1 levels, and respiratory parameters were measured on day 15.

Chronic ritanserin study

In an additional series of experiments, UAO and control rats were treated with a high dose of ritanserin to stimulate SWS and GH secretion. Animals were injected intraperitoneally with ritanserin (2 mg·kg^{-1}) or vehicle at lights-on for eight consecutive days [15].

Sleep

Data were collected on day 16 (DSI, St Paul, MN, USA) and sleep signals were scored using software (NeuroScore version 2.1; DSI) and edited visually. The duration of sleep–wake states were calculated in 1-h time blocks [8] and were categorised as wake, SWS and paradoxical sleep (PS). Power density during the first 3 h of light was assessed separately for each vigilance state [17]. The power density values for 0.5–4.0 Hz were integrated and used as an index of electroencephalogram slow-wave activity during NREM sleep [10]. The effect of ritanserin on slow-wave activity was calculated as the ratio of mean slow-wave activity obtained following ritanserin *versus* slow-wave activity obtained at baseline [17].

Hypothalamic mRNA expression

RNA was extracted and quantitative real-time PCR assays were performed [15] for GHRH, GHRHR, GH receptor, orexin and β-actin. Total RNA from two animals was pooled and assayed as one sample, yielding eight combined samples in each group.

Proteins

Proteins were detected by Western blot [14, 15] in a subset of seven sham and eight UAO rats on day 16.

Immunoassays

Serum GH and IGF-1 concentrations were measured using ELISA kits (DSL Inc., Webster, TX, USA) on day 16. Hypothalamic GHRH was determined by ELISA kit (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instructions on day 16.

Data analysis

Significance was analysed by unpaired t-test. One-way ANOVA was used to determine significance differences in hypothalamic GHRH following administration of ritanserin or vehicle. Two-way ANOVA for repeated measures was used to determine significance between time and group, or frequency and group, for *post hoc* comparisons by Student–Newman–Kuels test. The null hypotheses were rejected at the 5% level.

RESULTS

Further results can be found in the online supplementary material.

A total of 117 animals were included in this study; the final numbers of animals were 55 and 50 for the UAO and sham control groups, respectively. During the surgical procedure, the mortality rate of the UAO group was 10% and an additional 10% mortality was observed 2–5 days after surgery.

At days 13–16, the UAO rats' behaviour was similar to that of controls; they explored their cage and engaged in social activity, such as grooming. The UAO animals all demonstrated audible wheezing, especially after activity, but no signs of respiratory distress or gasping at rest were observed. As expected, following tracheal obstruction surgery, there were signs of increased mechanical load, *i.e.* inspiratory swings in oesophageal pressure (P_{oes}) more than doubled ($p=0.03$), respiratory rate decreased ($p<0.001$) and tracheal resistance ($n=8$ in each group) increased by 46% ($p=0.03$) (online supplementary table 1E). Arterial blood gases and haemoglobin ($n=8$ in each group) were normal and unchanged between groups (online supplementary table 1E). The growth rate was significantly lower in UAO animals compared with controls ($n=11$ in each group) (online supplementary table 2E). 13 days post-surgery, food intake was unchanged in the control ($n=10$) and UAO ($n=11$) rats (mean$\pm$SD 148$\pm$3.4 and 164$\pm$6.9 g food per kg body weight, respectively; $p=0.1$). Both the control ($n=8$) and UAO groups ($n=8$) exhibited circadian rhythms of body temperature. Mean$\pm$SEM 12-h light-period body temperature was 37.0$\pm$0.03 and 36.5$\pm$0.03°C for the control and UAO rats, respectively ($p<0.01$). During the dark period, 12-h body temperature was lower in UAO rats compared with controls (37.0$\pm$0.04 and 37.5$\pm$0.04°C, respectively; $p<0.01$).

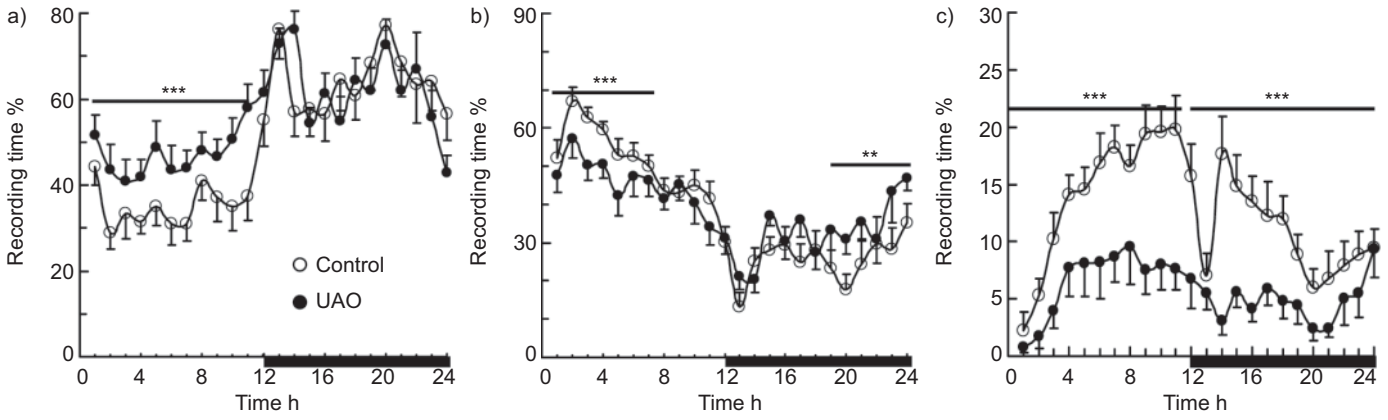

FIGURE 1. Spontaneous sleep in control and upper airway obstruction (UAO) rats. Hourly values of a) wake, b) slow-wave sleep (SWS) and c) paradoxical sleep (PS) are shown. Black horizontal bars represent the lights-off (active) period of a 12/12-h cycle with lights on at 09:00 h. UAO rats had significantly more wake, and less SWS and PS than controls during the light period. During the dark period, UAO rats had significantly more SWS and less PS than controls. Data are from 11 control and 11 UAO rats. Data are presented as mean±SEM. **: $p<0.01$; ***: $p<0.001$.

Sleep duration

Both the control (n=11) and UAO (n=11) rats exhibited circadian rhythms of wake, SWS and PS. As expected for nocturnal animals, there was more sleep in the light period than in the dark period (fig. 1). There were, however, several significant differences in sleep between UAO and control groups (fig. 1 and table 1). The UAO group was awake 36% more during the light period ($p<0.001$) and had 18% shorter SWS duration during the light period ($p<0.001$). UAO rats had significant elevation of light SWS (SWS-1) and reduction of deep SWS (SWS-2), compared with controls, during the first 6 h of the light period ($p<0.01$). Interestingly, during the dark period, UAO animals had 38.3% more SWS than controls ($p<0.001$). Obstructed animals had 41% less PS ($p<0.001$) than controls during both light and dark periods (fig. 1 and table 1).

To determine the total duration of each sleep–wake stage, we quantified the number and length of each sleep stage epoch (fig. 2) for the 12-h light periods. UAO animals had more frequently interrupted sleep compared with controls. The obstructed group had 35% more wake bouts and these were 67% longer than in controls ($p<0.001$). UAO rats manifested a 37% reduction in the number of SWS bouts ($p<0.01$) without any change in duration, and a 50% decrease in the number of bouts and duration of PS ($p<0.001$).

Power density analysis

During SWS, the power density of UAO rats (n=11) decreased by 40% at 1.5 Hz compared with controls (n=11) (online supplementary fig. 1E; $p<0.001$). Spectral analysis showed that SWS-1 and SWS-2 decreased by 37 and 57%, respectively, in the UAO group during the first 3 h of the light period, in the frequency range of 0.5–4 Hz, compared with controls (data not shown; $p<0.001$). The time-course of electroencephalogram slow-wave activity during NREM sleep showed a normal pattern in the controls, with high slow-wave activity values at the beginning of the rest period followed by a decline towards dark onset and a gradual increase during the dark period (fig. 3). In contrast, the daily course of electroencephalogram slow-wave activity was flat in UAO rats; there were no significant changes detected in slow-wave activity depth over the course of the day ($p<0.001$).

Ritanserin study

Acute administration of the a high dose of the 5-HT_2 receptor antagonist ritanserin significantly decreased ($p<0.01$) wake duration during lights-on in UAO rats (n=10 in each group) to levels statistically similar to those of controls (fig. 4). In both groups, ritanserin significantly increased SWS duration $\leqslant$6 h following drug administration; this was related to elevation of SWS-2 duration (fig. 4 and table 1). Ritanserin increased slow-wave activity $\leqslant$6 h following drug administration in both groups ($p<0.01$; fig. 3b). Confirming earlier reports [15], during the chronic ritanserin study, growth gain parameters were ~50% lower ($p<0.001$) in UAO rats (n=12) compared with controls (n=10). Ritanserin partially normalised somatic growth retardation, as revealed by tail and tibial lengths, but not body weight gain (fig. 5). UAO decreased serum IGF-1 in rats treated with vehicle (mean±SEM 700±80 ng·mL^{-1}) compared to UAO

TABLE 1 Spontaneous sleep values during 12-h lights-on period

	Control#		UAO#	
	Baseline	Ritanserin	Baseline	Ritanserin
Wake	38.9±2.3	33.6±2.2	52.9±2.6**	41.1±2.3***
SWS	45.3±1.6	52.0±1.9***	37.1±2.6**	48.7±1.8***
PS	15.7±1.8	14.6±0.8	9.2±1.2**	10.6±1.0***
SWS-1	31.8±1.0	31.1±0.9	29.1±2.2	29.2±1.5
SWS-2	13.7±1.3	21.0±2.1***	8.4±2.2**	19.0±1.4***

Data are presented as mean±SEM % of time spent in each sleep stage for the light period (09:00–21:00 h). UAO: upper airway obstruction; SWS: short-wave sleep; PS: paradoxical sleep; SWS-1: light SWS; SWS-2: deep SWS (stages 3–4 in humans). #: n=10. **: $p<0.01$ comparing baseline (vehicle) control with baseline (vehicle) UAO by two-way ANOVA; ***: $p<0.001$ comparing ritanserin (2 mg·kg^{-1}) with baseline (vehicle) by two-way ANOVA.

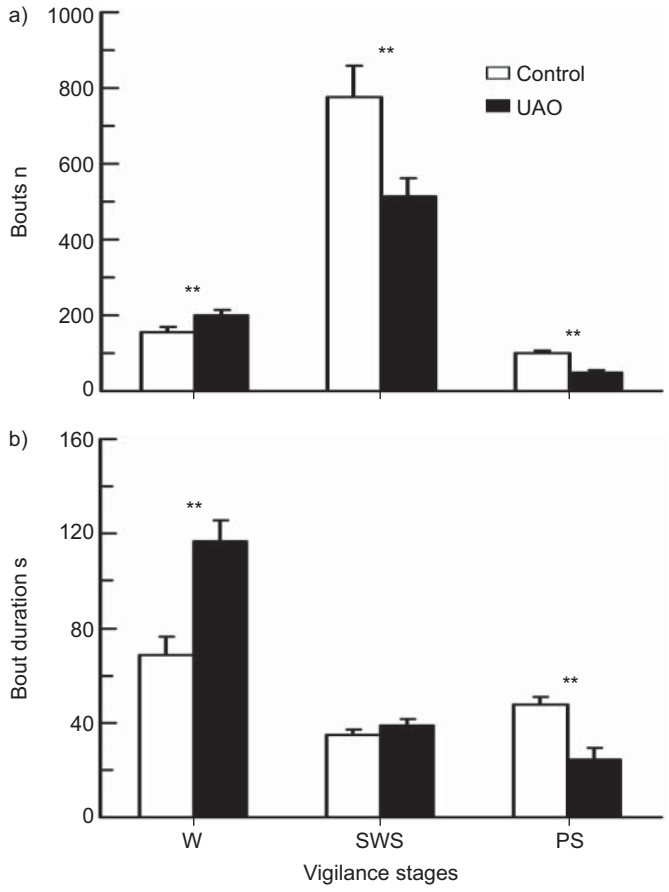

FIGURE 2. a) Total number and b) duration of each sleep–wake stage during the 12-h lights-on period. Data are presented as mean±SEM. W: wake; SWS: slow-wave sleep; PS: paradoxical sleep; UAO: upper airway obstruction. **: $p<0.01$.

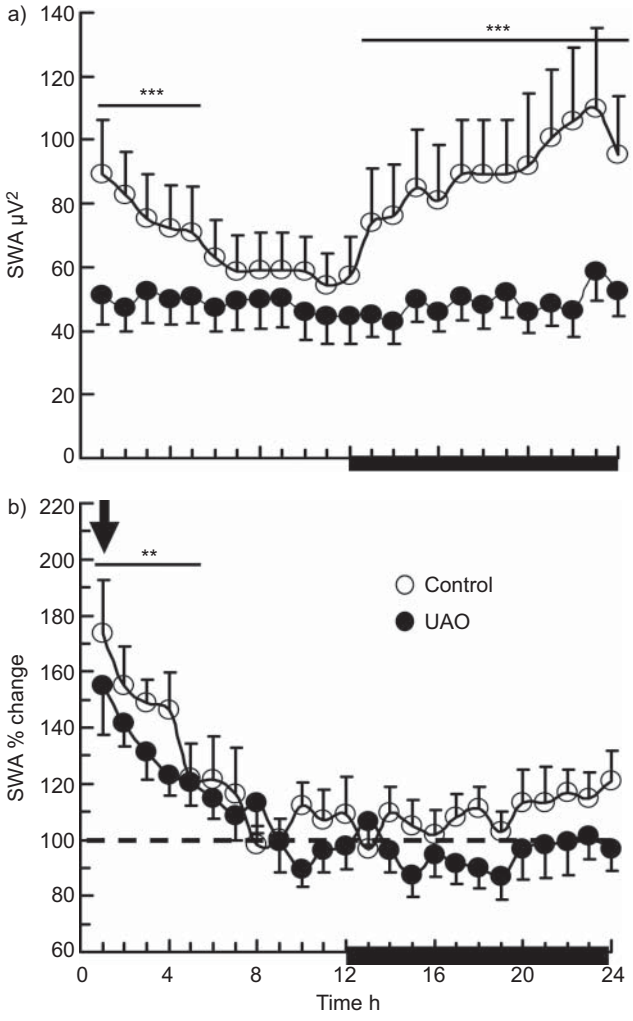

FIGURE 3. a) Hourly average of electroencephalogram slow-wave activity (SWA; integrated power densities in delta range). SWA was significantly lower in upper airway obstruction (UAO) rats compared with controls. b) Effect of ritanserin (2 mg·kg^{-1}) on SWA calculated as percentage of baseline values on day 15 (arrow identifies time of injection). Black horizontal bars represent lights-off on a 12/12-h cycle with lights on at 09:00 h. Data are presented as mean±SEM. **: $p<0.01$ following ritanserin relative to baseline vehicle values on day 15 in both groups; ***: $p<0.001$ between groups.

treated with vehicle (550±50 ng·mL^{-1}) ($p=0.01$). Ritanserin normalised this effect on serum IGF-1 values (680±90 ng·mL^{-1}; not significantly different from the control group).

Endocrine analysis

Endocrine analysis was performed in a subset of 10 sham and 12 UAO rats: serum GH (101±8 ng·mL^{-1} in control and 62±5 ng·mL^{-1} in UAO rats; $p=0.038$) and IGF-1 level (1,400±86 ng·mL^{-1} in control and 870±60 ng·mL^{-1} in UAO rats; $n=9$; $p=0.001$) were significantly reduced in UAO. Hypothalamic GHRH mRNA (fig. 6a) and protein (fig. 6b) decreased in UAO animals by 30% ($p=0.002$) and 25% ($p=0.001$), respectively. Hypothalamic GHRHR protein decreased by 72% in UAO animals ($p=0.001$) (fig. 6c). Administration of ritanserin for 8 days at lights-on normalised hypothalamic GHRH content in UAO animals (controls 1.6±0.29 pg GHRH per mg tissue; UAO animals 1.5±0.19 pg GHRH per mg tissue; $p=0.327$, one-way ANOVA; $n=10$ in each group). Hypothalamic orexin mRNA increased by 73% in UAO animals ($p<0.01$) (online supplementary fig. 3E).

DISCUSSION

We have shown that growth retardation in UAO is associated with reduction of hypothalamic GHRH content, which could explain both the abnormal SWS and impaired GH homeostasis in juvenile UAO rats. Our data suggest that a similar mechanism could be responsible for growth retardation of children suffering from UAO without preserved sleep architecture.

It is not clear why growth retardation occurs only in 5–25% of children with sleep-disordered breathing [1–4]. Several controversial mechanisms have been suggested to explain this phenomenon: dysphagia and decreased appetite [4], increased work of breathing during sleep [3], and abnormal GH homeostasis [1, 2]. In this study, we offer the suggestion of the involvement of an impaired GH axis as a major mechanism of growth retardation. In contrast to our UAO rats, children with UAO typically do not have an elevated arousal index secondary to obstructive events and have preserved sleep stage durations [18]. Our results may suggest that growth retardation may occur in a subset of children without preserved sleep architecture.

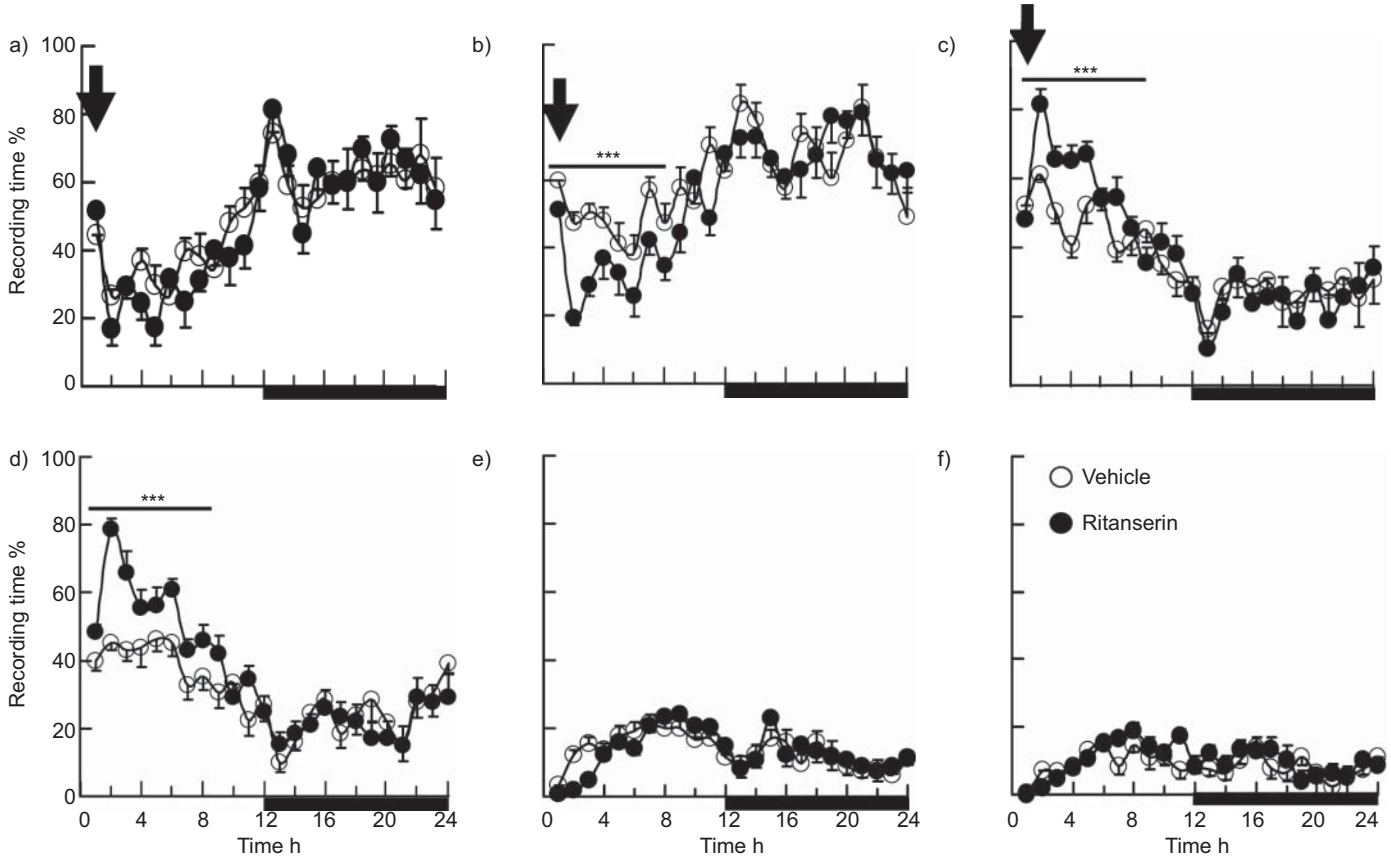

FIGURE 4. Effect of ritanserin (2 mg·kg^{-1}) on spontaneous sleep. Hourly values of a, b) wake, c, d) slow-wave sleep and e, f) paradoxical sleep are shown in a, c, e) control and b, d, f) upper airway obstruction rats. Ritanserin (2 mg·kg^{-1}; baseline: day 16) or vehicle (baseline: day 12) was administered at lights-on (arrow). Black horizontal bars represent the lights-off (active) period in a 12/12-h cycle with lights on at 09:00 h. Data are presented as mean±SEM. ***: $p<0.001$ between vehicle and ritanserin values.

We have also shown that pharmacological correction of the abnormalities in the GHRH/GH axis may potentially be a promising strategy for therapeutic interventions designed to improve growth in affected children.

Strengths and limitations of the model

Chronic upper airway loading was induced in 22-day-old rats and animals were followed for 2 weeks, a period comparable to 6 months to 8 yrs of age in children. Following surgery, inspiratory swings in P_{oes} and tracheal resistance increased, consistent with increased airway resistive loading. Previously, we demonstrated that UAO led to a decreased respiratory rate and tidal volume accompanied by mild elevation of arterial carbon dioxide tension (P_{CO_2}) [15, 19]. Because our measurements of P_{oes} were taken under anaesthesia, they may underestimate the true effects of obstruction on intrathoracic pressure. The unchanged P_{CO_2}, and reduced respiratory rate and P_{oes} in the current study suggest that the trachea was not

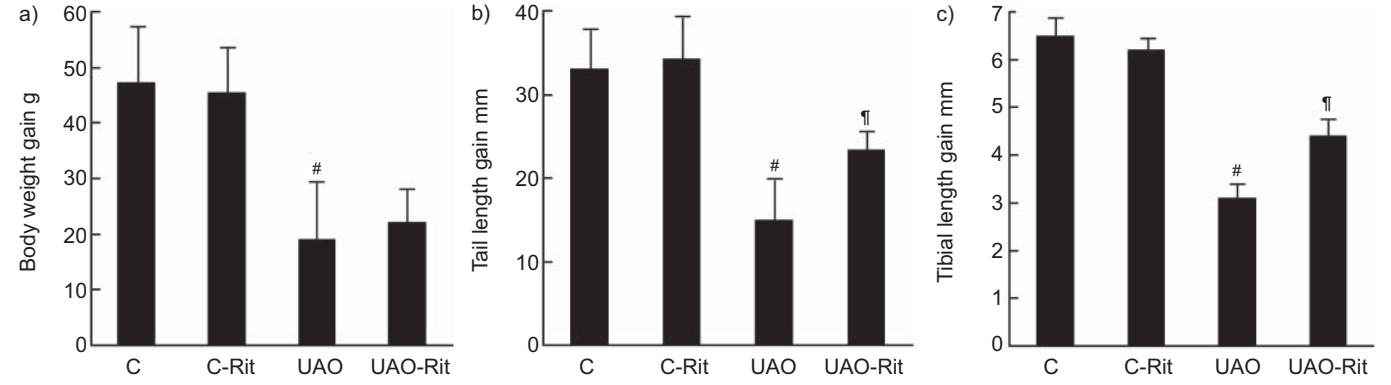

FIGURE 5. Effect of ritanserin on somatic growth parameters. a) Body weight gain, b) tail length gain and c) tibial length gain. C: control; C-Rit: control plus ritanserin (2 mg·kg^{-1}); UAO: upper airway obstruction; UAO-Rit: upper airway obstruction plus ritanserin. Data are presented as mean±SD. #: statistically significant for C *versus* UAO; ¶: statistically significant for UAO *versus* UAO-Rit.

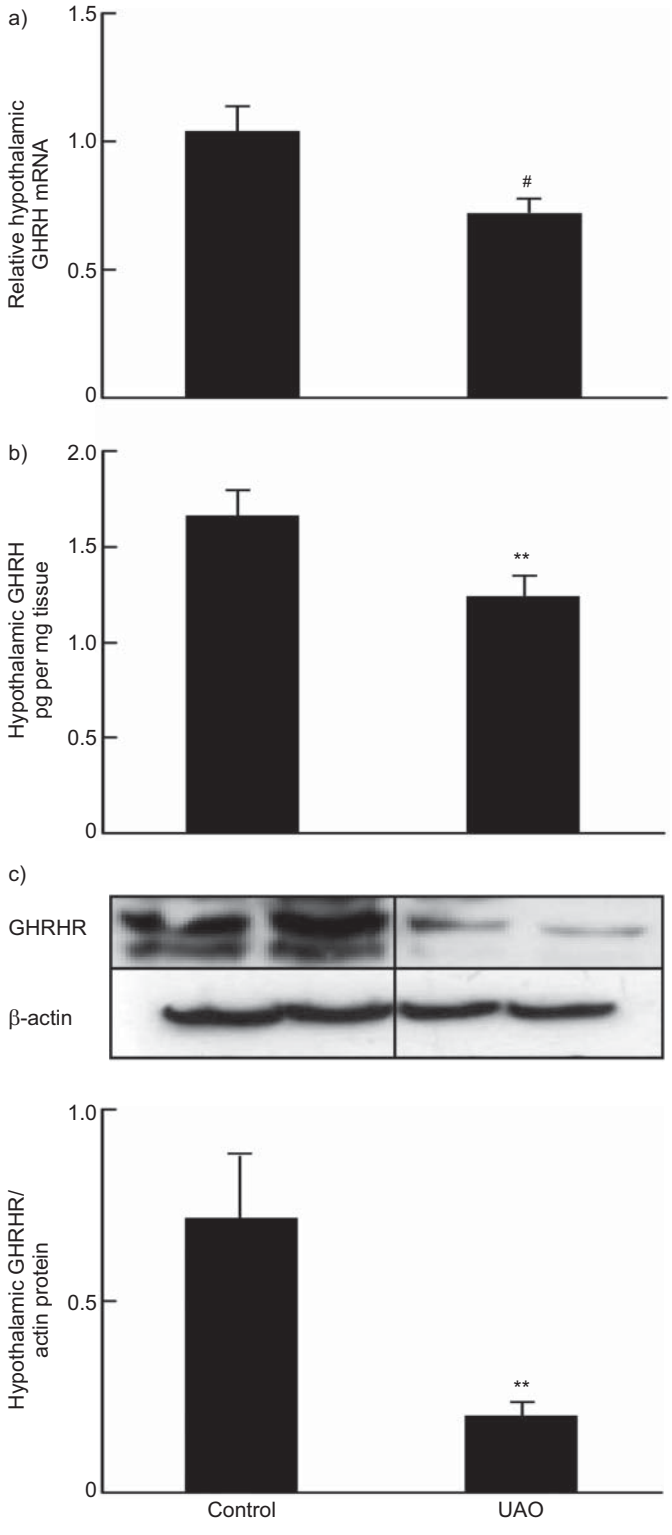

FIGURE 6. a) Relative hypothalamic growth hormone-releasing hormone (GHRH) mRNA level determined by real-time PCR. b) Hypothalamic GHRH content, determined by ELISA. c) Representative Western blot analysis of hypothalamic GHRH receptor (GHRHR) and densitometric analysis of GHRHR protein. UAO: upper airway obstruction. #: p=0.002; **: p=0.01.

severely obstructed and could be considered typical for mild-to-moderate respiratory loading. Under these conditions, animals were able to maintain normal resting ventilation and arterial oxygen tension [14, 15, 19]. In children, however, oxygen saturation usually decreases with airway loading during sleep [20]. The UAO model has been used to explore adaptive changes in respiratory system function [14, 15, 19, 21, 22]. In this model, both inspiratory and expiratory loading were introduced, which may resemble subglottic stenosis in children and not be exclusively sleep related, while in clinical sleep-disordered breathing, airway loading is mainly inspiratory and sleep related [18]. Obstructive sleep apnoea is associated with intermittent UAO at night, primarily during inspiration. It seems likely that our model also has implications for this condition since, as in sleep apnoea, the animals exhibited sleep fragmentation and, as in children with sleep apnoea, the animals showed growth retardation.

Ritanserin treatment normalised the GH axis and growth parameters were partially restored. This finding may suggest that, in addition to GH and GHRH, other mechanisms may also be involved in growth retardation in this model. It is possible that there is impairment of total body energy balance related to increased work of breathing [3] and/or increased energy expenditure due to enhanced locomotion activity due to the sleep fragmentation. There is little evidence to support these possibilities, as total body energy in UAO rats during quiet wakefulness was similar to that in controls [14]. Mean locomotor activity in UAO rats was unchanged or even decreased during active and quiet phases of the day, respectively [15]. Finally, protein loss and cachexia may have been induced following increased production of oxygen-derived free radicals and cytokines, as a result of strenuous contractions of respiratory muscles associated with upper airway loading [23]. However, previous studies have not found changes in serum pro-inflammatory factors, such as interleukin-6 and tumour necrosis factor-α, in UAO rats [24].

UAO and GHRH/GH axis

Chronic UAO inhibited the secretion of hypothalamic GHRH in our model. Hypothalamic GHRH is necessary to maintain GHRHR synthesis and both GH and GHRH regulate their own synthesis by negative feedback at the level of the hypothalamus [9, 25–27]. In order to better understand the distribution and localisation of GHRH and GHRHR in specific hypothalamic nuclei, future studies should be performed using immunocytochemical staining. In our study, PS was significantly reduced in UAO rats. PS deprivation can significantly decrease GHRH mRNA in the paraventricular and arcuate nuclei [28].

Orexins/hypocretins are neuropeptides inhibiting GHRH in hypothalamic nuclei involved in NREM and rapid eye movement sleep regulation [29]. In UAO rats, orexin mRNA increased by 73%, possibly as a result of forced physical activity [21] and/or increased awakening [30]. Interestingly, the neuroendocrine effects that we observed in UAO rats resemble those seen following prolonged sleep-deprivation/restriction in rats, including decreased body temperature [31], depletion of hypothalamic GHRH content [7, 8] and reduction of serum GH [32]. It is possible that sleep fragmentation due to UAO leads to orexin increase and abnormal serotonergic

balance, which results in reduced SWS and GHRH [29]. Further studies are needed to explore this issue.

The strong association between NREM sleep and GH release raises the possibility that pharmacological agents that increase NREM sleep can also stimulate GH secretion [13]. In the current study, hypothalamic GHRH/GH axis and sleep in UAO rats were reversed by administration of ritanserin. Stimulation of GH secretion and promotion of sleep are two closely interrelated outputs of hypothalamic GHRHergic neurons [9]. As in our study, administration of a high dose of ritanserin had a strong sleep consolidation effect in rats [33] and in humans with pre-existing sleep fragmentation [34]. This could provide additional explanation for the improved sleep duration and slow-wave activity following ritanserin administration in UAO. Finally, similarly to other reports [33, 35], the effects of ritanserin on SWS and wake cycles are limited to the first hours of light onset following drug administration due to its known pharmacokinetics.

We found that decreased GHRH was associated with both sleep and growth impairment. This is consistent with studies showing that sleep duration and depth are suppressed when GHRH is inhibited by means of a competitive GHRHR antagonist, activation of the negative feedback in the somatotropic axis or after somatostatinergic stimulation [7, 8]. Slow-wave activity is also regulated, in part, *via* intrinsic cortical GHRH [36]. Slow-wave activity increases during wakefulness, due to cortical and motor activities during awakening, and declines during sleep [37]. Indeed, consistent with this, our UAO rats spent significantly more time in SWS (fig. 1b) during the dark phase and were less active during the dark phase [15]. Further studies are needed to explore the role of cortical GHRH in juvenile UAO rats.

Several possibilities may explain the reduction of PS in our UAO rats. During PS, the skeletal muscle force is minimal and insufficient to support ventilation during chronic resistive loading, and the arousal threshold to acoustic stimulation is lower [38], resulting in shortening of this stage in our rats. Our UAO animals had lower GH levels. Rats with impairment in GHRH signalling have less PS than normal rats [39].

Conclusion

This study presents evidence that growth retardation in UAO is associated with a reduction in hypothalamic GHRH content. Our findings show that the abnormalities in the GHRH/GH axis underlie both growth retardation and SWS-disorder UAO.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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