Journal of Integrative Neuroscience

Short Communication

Altered production of reproductive neuropeptides in rats subjected to chronic intermittent hypoxia

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DOI:10.31083/j.jin2003069

Submitted: 15 April 2021 Revised: 30 April 2021 Accepted: 28 May 2021 Published: 30 September 2021

Hypobaric hypoxia is a stressful condition known to decrease fertility both in humans and animals. However, the mechanism by which the hypothalamus-pituitary-gonad axis is altered remains unknown. The aim of the present study was to analyze the effects of chronic intermittent and continuous exposure to hypoxia on hypothalamic-pituitary-gonadal axis regulation in male rats. Thirty adult male Wistar rats were assigned to one of the following three groups: control group; chronic intermittent hypoxia: subjected to 600 mbar for 18 h/d five days a week; and chronic continuous hypoxia: subjected to 600 mbar for 23.5 hours/day seven days a week, for 30 days. Plasma luteinizing hormone and testosterone concentration, hypothalamic *GnRh*, *Kiss1* and *Rfrp3* mRNA levels and PGE_2 content were determined. Levels of *Rfrp3*, a negative regulator of GnRH and LH release, were higher in intermittently exposed animals than in controls. Levels of Kiss1, a neuropeptide that stimulates the release of GnRH only increased in animals exposed to continuous hypoxia. Plasma luteinizing hormone and testosterone concentrations and body weight were lower in rats subjected to intermittent hypoxia as compared to the remaining groups. *GnRh* mRNA levels as well as PGE_2 content remained unchanged in all groups. Taken together, results suggest that besides the well documented direct effects of hypoxia on the testes, infertility observed in male rats exposed to hypoxia may also be due to overexpression of negative regulators of GnRH and luteinizing hormone release. Intermittent, rather than continuous, to hypoxia exposure would seem to be more detrimental to fertility.

Keywords

Hypoxia; Hypothalamic peptides; Hypothalamic-pituitary-gonadal axis

1. Introduction

Reproductive functions in adults are mainly controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormone (GnRH) secretion by specific hypothalamic neurons is regulated by several factors: gonadal steroids, neurotransmitters (including dopamine, GABA, histamine, and opioids) and by hypothalamic neuropeptides, such as gonadotropin-inhibitory hormone (GnIH)/RFamide-related peptides (Rfrp) and kisspeptin, a peptide encoded by the *Kiss1* gene [1]. Rfrp3 is known to suppress the HPG axis at the level of both the hypothalamus and adenohypophysis (AH) inhibiting GnRH and luteinizing hormone (LH) secretion [2, 3]. Kisspeptin is a stimulatory neuropeptide and therefore increases GnRH synthesis and secretion [4]. There is evidence that Rfrp-3 may affect the signaling to GnRH neurons from other neuronal fibers, such as kisspeptin neurons, indicating that there exists an intricate balance between inhibitory and excitatory neuronal signals that ultimately modulate the activity of the reproductive axis [5]. In addition to the neuropeptides mentioned above, prostaglandin E_2 (PGE_2) is also a positive regulator for HPG axis secretion, as shown by studies reporting its stimulatory effects on GnRH and LH [6, 7].

GnRH is released from its neuronal hypothalamic terminals to the portal vessels and reaches the AH, a specific region of the pituitary gland, where it induces the release of LH and follicle-stimulating hormone (FSH) into the bloodstream. LH is responsible for stimulating sex steroid secretion (such as testosterone (T), estradiol, and progesterone) from the gonads of both sexes, whereas FSH is the main gametogenic hormone [8].

Hypoxia (HX) is considered a stressful stimulus and the body must develop compensatory physiological responses in order to ensure homeostasis. Continuous HX is experienced by populations living at high altitude and who, as a result, have undergone phenotypic and genetic adaptations to cope with low environmental O_2 [9]. Exposure to intermittent HX is also frequent, as is the case for tourists traveling to high altitude destinations, people engaged in certain work activities and people who suffer a systemic alteration such as obstructive sleep apnea, heart failure and pulmonary conditions [10, 11]. A number of studies have shown exposure to hypoxic conditions decreases fertility in both male and female animals and humans. Decreased LH and T levels have been reported in males with OSA [12] and in males staying at 3,500 meters above sea level for seven days [13]. Furthermore, animal studies have shown the same hormonal patterns in male rats as those observed in human males [14], as well as the occurrence of changes in testicular morphology, metabolic stress and loss of spermatogenic cells [15, 16]. De-

spite the deleterious effect of hypoxic exposure on HPG axis hormone secretion, the mechanism by which this occurs remains unclear. Reports in the literature are not conclusive as to whether intermittent or continuous exposure to HX is more detrimental to fertility.

The present study sought to analyze the effects of chronic intermittent and continuous exposure to hypoxia on the mechanisms that modulate HPG axis activity. The aim is to contribute to understanding of hypoxia-related male infertility.

2. Materials and methods

2.1 Experimental design

Male Wistar rats (250–300 g) supplied by the animal housing facility of the School of Pharmacy and Biochemistry, University of Buenos Aires (Argentina), were used throughout. Animals had free access to food and water and were kept under controlled light (12 h light/dark) and temperature (20–25 °C) conditions. The broad temperature range is explained by the heat released during normal functioning of the hypoxic chambers. All animal procedures were performed according to the NIH Guide for the Care and use of Laboratory animals (NIH 8th edition, 2011). Protocols were approved by the institutional Animal Care and Use Committee (IACUC, N^o 11/06/2012-23) of the School of Dentistry, University of Buenos Aires. Rats were randomly assigned to one of three groups of ten animals each subject to the following conditions: Control group (C): normoxia; Chronic Intermittent Hypoxia (CIH): exposure to discontinuous hypoxia (600 mbar, equivalent to 4,000 meters of simulated altitude) in a simulated high altitude chamber for 18 h/d, five days a week for 30 days (time of exposure to hypoxia was approximately 50 % of the experimental period); Chronic Continuous Hypoxia (CCH): continuous exposure to the same ambient pressure in the simulated high altitude chamber as the CIH group but for 23.5 h/d seven days a week for 30 days (time of exposure to hypoxia was almost 100 % of the experimental period) [17]. Environmental oxygen pressure was 12.6 kPa during the hypoxic period in both hypoxic protocols. Animals were euthanized by decapitation. Trunk blood samples were collected in chilled heparinized tubes, centrifuged at 3,625 g at 4 °C for 20 min and the plasma separated and stored frozen at -20 °C for biochemical analysis. Another set of trunk blood samples was collected in heparinized microcapillary tubes and centrifuged at 1,790 g for microhematocrit analysis.

2.2 Testosterone determination

Plasma T concentration was determined using a rat enzyme-linked immunosorbent assay (ELISA) with antibodies and standards from DRG Instruments (GmbH, Marburg, Germany), as described by Surkin *et al.*, [1]. Absorbance was determined at 450 nm on a microplate reader (Model 3550, BIO-RAD Laboratories, California, USA). Plasma T levels are expressed as ng/mL.

2.3 Radioimmunoassay

2.3.1 PGE_2

As hypothalamic PGE_2 acts as a gliotransmitter that regulates the activity of GnRH-releasing neurons and pituitary PGE_2 and has been reported to be a potent stimulator of LH secretion, PGE_2 content was analyzed in both organs. To that end, the hypothalamus and pituitary gland were homogenized in ice cold ethanol (100 %), centrifuged at 10,000× g min at 4 °C for 15 min, and the supernatant collected and evaporated in a centrifugal vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Waltham, MA). Residues were resuspended in radioimmunoassay buffer and Sigma antiserum was used (Sigma-Aldrich, St. Louis, MO, USA). PGE2 content is expressed as pg/mg of weighed tissue [18].

2.3.2 LH

Plasma LH was measured by RIA using rat LH antiserum (NIDDK-anti-rLH-S-II), antigen (NIDDK-rLH-I) and reference preparation (NIDDK-rLH-RP-3) purchased from Dr. A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases; Torrance, CA, USA). All samples were measured in duplicate; results are expressed as ng of LH/mL of plasma [19].

2.4 Polymerase chain reaction

Following euthanasia, hypothalami were harvested in RNAZOL Reagent and frozen at –80 °C until use. Total RNA was isolated from cells according to the manufacturer's instructions (Molecular Research Center Inc., Cincinnati, OH, USA). After RNA extraction, genomic DNA was digested with DNAse at room temperature for 10 minutes. DNAsa was then inactivated and RNA was quantified by NanoDrop (Eppendorf, Hamburg, Germany). cDNA was synthesized from total RNA (3 μg) using M-MLV reverse transcriptase, random primers and ribonuclease inhibitor. The specific primers to perform PCR amplification were designed using Primer 3 Software and the sequences were GAPDH: forward 5′-TGTGAACGGATTTGGCCGTA-3′ and reverse 5′-ATGAAGGGTCGTTGATGGC-3′, product (bp): 92, *GnRH*: forward 5′-GCCGCTGTTGTTCTGTTGAC-3′ and reverse 5′-TCCTCCTTGCCCATCTCTTG-3′, product (bp) 131, *Rrpv-3*: forward 5′-CTGAACCCACAGGCCAACAGT-3′ and reverse 5′-AAGGAGTTCCAGTTGTAGGCTG-3′, product (bp) 93, and *Kiss-1* forward 5′-CTGCTGCTTCTCCTCTGTG-3′ and reverse 5′-CCCCAGGCTTGCTCTCTG-3′, product (bp): 154. The following settings were used for each gene: 94 °C for 5 min; 35 cycles of 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 1 min; and 72 °C for 5 min. The products were loaded onto 2% agarose gel, and bands were visualized on a transilluminator under UV light. Photographs were taken with a digital camera (Olympus C-5060, Tokyo, Japan) and analyzed using Image Lab software (Bio-Rad Laboratories, California, USA). The relative mRNA level was normalized to GAPDH; results are expressed as arbitrary units of relative optical density [17].

Table 1. Body weight and hematocrit of male rats exposed to hypoxia for 30 days.

	Control	CIH	CCH
Body weight (g)	512.50 ± 25.88^a	399.4 ± 11.78^b	487.30 ± 28.18^a
Hematocrit (%)	42.6 ± 2.30^a	52.4 ± 3.50^b	56.6 ± 2.51^b

C, control; CIH, chronic intermittent hypoxia; CCH, chronic continuous hypoxia. Results expressed as mean ± SEM. Different letters represent statistical significance, $p < 0.05$, $n = 5$ in each group.

2.5 Statistical analysis

Results are expressed as box plots, where the lower, central, and upper sides of the box represent the first, second (median), and third quartile of the data points, respectively; the lower and upper whiskers represent the lower and upper value of the data, respectively. All data were processed using Infostat (Córdoba, Argentina). The significance of the differences between means was determined by one-way ANOVA followed by Tukey's test. Statistical significance was set at $p < 0.05$. All experiments and corresponding determinations were performed in triplicate; figures give the results of one experiment.

3. Results

Following the findings reported by Farias *et al.* [14], body weight at the end of the experimental period was lower in rats exposed to intermittent hypoxia than in the other two groups ($F_{1,14} = 32.71$; $p < 0.0001$). As expected, hematocrit was higher in hypoxic rats than in controls ($F_{1,14} = 31.89$; $p < 0.0001$) (Table 1).

3.1 Intermittent exposure to hypoxia for 30 days decreased LH and testosterone concentration

Analysis of hormonal secretion by the HPG axis showed lower LH and testosterone concentration in rats exposed intermittently to hypoxia than in controls (LH: C: 0.68 ± 0.14, CIH: 0.46 ± 0.13, CCH: 0.58 ± 0.17; $F_{1,28} = 3.80$; $p = 0.035$; T: C: 4.95 ± 2.16, CIH: 2.60 ± 1.39, CCH: 3.26 ± 0.97; $F_{1,20} = 4.19$; $p = 0.032$). Hormone levels were slightly lower in CCH rats as compared to controls, although the difference did not reach statistical significance. Interestingly, *Gnrh* mRNA levels did not vary significantly among the groups (C: 0.18 ± 0.08, CIH: 0.18 ± 0.05, CCH: 0.17 ± 0.05; $F_{1,17} = 0.76$; $p = 0.93$) (Fig. 1).

3.2 Intermittent hypoxic exposure resulted in an increase in Rfrp3 mRNA levels

Analysis of the neural factors that regulate HPG axis activity showed higher *Rfrp3* mRNA levels in hypothalamic neurons of the CIH group compared to those observed in the other two groups (C: 0.14 ± 0.09, CIH: 1.41 ± 0.65, CCH: 0.41 ± 0.12; $F_{1,16} = 5.56$; $p = 0.016$). This suggests that the lower LH levels observed in rats intermittently exposed to hypoxia could be due to the inhibitory action of Rfrp3 on gonadotropin releasing cells in the AH. Conversely, *Kiss1* mRNA levels increased in the CCH group only (C: 0.06 ± 0.07, CIH: 0.04 ± 0.04, CCH: 0.19 ± 0.16; $F_{1,18} = 4.79$; $p = 0.023$) (Fig. 2).

3.3 Hypoxia did not seem to affect hypothalamic and pituitary PGE_2 levels

Hypothalamic PGE_2 and pituitary PGE_2 analysis showed that hypoxia did not affect PGE_2 levels in either of the studied tissues (hypothalamic PGE_2 levels: C: 793.39 ± 182.97, CIH: 733.62 ± 119.72, CCH: 831.21 ± 301.80; $F_{1,18} = 0.1$; $p = 0.90$; pituitary PGE_2 levels: C: 30.99 ± 9.33, CIH: 33.21 ± 12.38, CCH: 30.84 ± 12.53; $F_{1,17} = 0.01$; $p = 0.98$) (Fig. 3).

4. Discussion

The results obtained in this study show that intermittent exposure to hypoxia increased the levels of *Rfrp3* mRNA, encoding gonadotropin-inhibitory hormone synthesis, which is consistent with the lower plasma LH levels observed for this same group. The lower levels of LH would likely explain the lower T levels observed in the CIH group. To the authors knowledge, this is the first study to explore the neuropeptides that regulate HPG axis secretion under the main types of hypoxic conditions commonly affecting humans.

Findings regarding the reported effects of hypoxia on human and animal fertility are controversial. In line with studies that show no change in T levels in HX-exposed rats as compared to rats maintained under normoxic conditions [20], Nelson *et al.*, [21] found no difference in LH or FSH levels between hypoxic animals and controls. However, human and animal studies, see Sawhaney *et al.*, [13], Gasco *et al.* [16], Farias *et al.*[17], Richalet *et al.* [22], and Liu *et al.* [23] showed a decrease in LH, FSH and T levels. In keeping with the former reports, the present study showed intermittent HX to result in lower LH, and consequently in lower T secretion. Of note, CCH animals showed similar LH and T levels to those of controls. Based on reported studies, it could be posited that hormonal response of the HPG axis varies according to the type of hypoxic exposure (i.e., intermittent or continuous), duration of exposure (a few days or months) and the altitude to which the subjects are exposed. Previous studies have shown that intermittent exposure to hypoxia HX has a more deleterious effect on tissues than continuous exposure [17, 24]. The present results would seem to indicate that the same occurs in the case of the HPG axis. Continuous exposure would allow the body to adapt and develop mechanisms to compensate for the lower oxygen levels, i.e., enhanced differentiation of neuron non-proliferating precursors [25]. Conversely, intermittent exposure would not allow the proper cellular signals to occur, leading to a failure in the acclimation process [26].

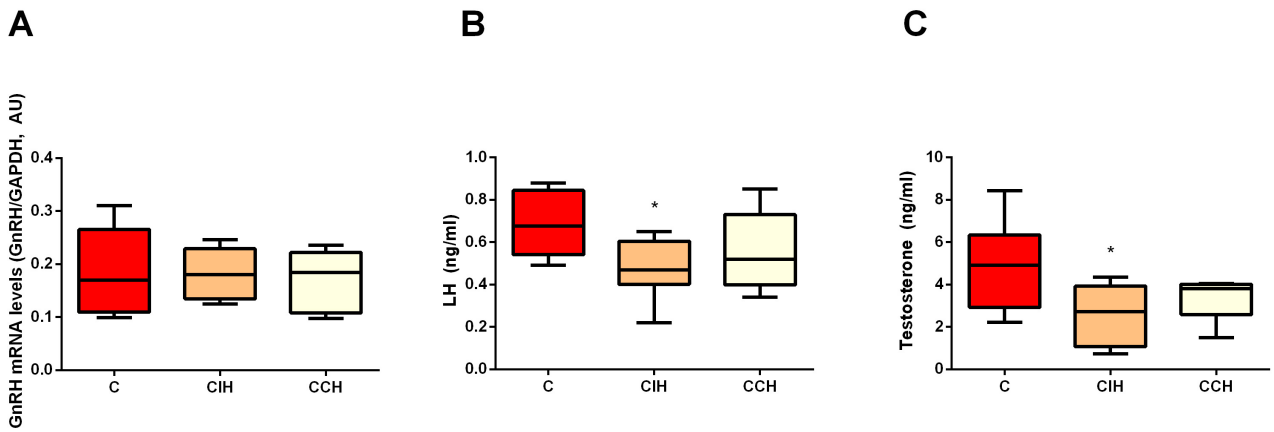

Fig. 1. Intermittent exposure to hypoxia for 30 days decreased LH and Testosterone concentration. Hypothalamic *GnRH* mRNA levels (A), LH concentration (B), and Testosterone concentration (C) in rats exposed to chronic hypoxia for 30 days. C, control; CIH, chronic intermittent hypoxia; CCH, chronic continuous hypoxia. Results are shown as boxplots. $*p < 0.05$ *vs.* C. $n = 7$ in each group. Results were analyzed using one-way ANOVA followed by Tukey's test.

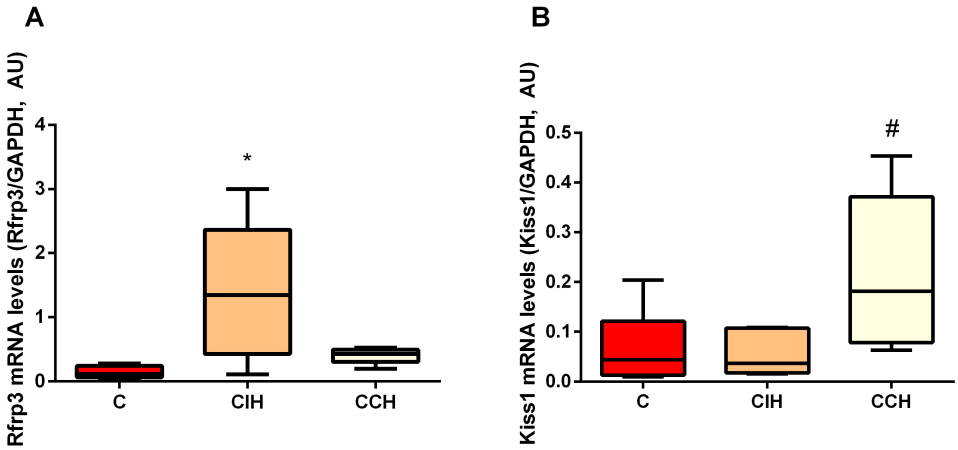

Fig. 2. Intermittent hypoxic exposure resulted in increased *Rfrp3* mRNA levels. Hypothalamic *Rfrp3* (A) and *Kiss1* (B) mRNA levels in rats exposed to chronic hypoxia for 30 days. C, control; CIH, chronic intermittent hypoxia; CCH, chronic continuous hypoxia. Results are shown as boxplots. $*p < 0.05$ *vs.* C and CCH, $\#p < 0.05$ *vs.* C and CIH. $n = 6$ in each group. Results were analyzed using one-way ANOVA followed by Tukey's test.

GnRH and LH release in males is subject to a negative feedback control mechanism that involves T [8]. The results presented here show lower T levels in CIH-exposed animals. Hence, altered LH and GnRH levels may also be expected in this group. However, although lower LH levels were found in CIH rats than in controls, *Gnrh* mRNA levels remained unchanged. The latter finding could indicate that CIH does not affect GnRH synthesis. Nevertheless, the potential occurrence of posttranscriptional changes, which would ultimately affect GnRH synthesis and secretion, cannot be ruled out. Additionally,hormonal feedback is only one of the several intricate mechanisms that control the activity of the HPG axis. As mentioned previously, GnRH synthesis and release are also regulated by signals from neurons and glial cells. Two of the main neuropeptides that regulate GnRH release are Rfrp3 (negative regulator of GnRH and kisspeptin (stimulator of GnRH neurons). The higher levels of *Rfrp3* found in the hypothalamic neurons of CIH animals would explain, at least in part, the lower levels of LH observed in this group, since Rfrp3 neurons are known to negatively regulate GnRH and kisspeptin release at the central level and LH release from the AH. Conversely, *Kiss1* mRNA levels were higher in continuously exposed rats, likely due to the absence of the inhibitory effect of Rfrp3 neurons. This may account for CCH animals showing similar LH and T levels to those of controls. Furthermore, given that PGE_2, mainly astrocyte PGE_2, triggers GnRH release [8] and hypoxia increases astrocyte PGE_2 production [27], hypothalamic PGE_2 levels were measured. Results show that PGE_2 levels remained unchanged in all the studied groups. This may be related to the fact that astrocyte production of PGE_2 is regulated by several molecules and is affected by different conditions besides hy-

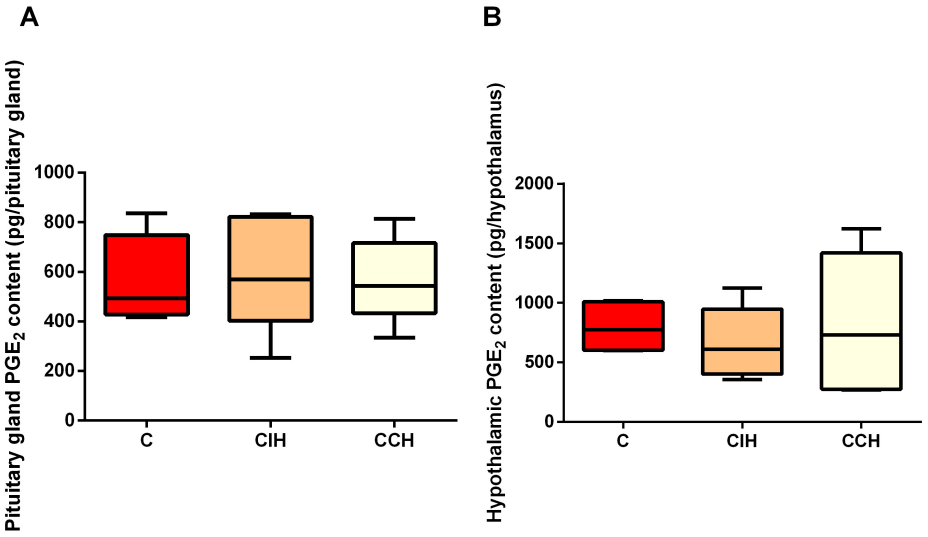

Fig. 3. Hypoxia did not seem to affect hypothalamic and pituitary PGE_2 levels. Pituitary (A) and hypothalamic (B) PGE_2 content in rats exposed to chronic hypoxia for 30 days. C, control; CIH, chronic intermittent hypoxia; CCH, chronic continuous hypoxia. Results are shown as boxplots; $n = 6$ in each group. Results were analyzed using one-way ANOVA followed by Tukey's test.

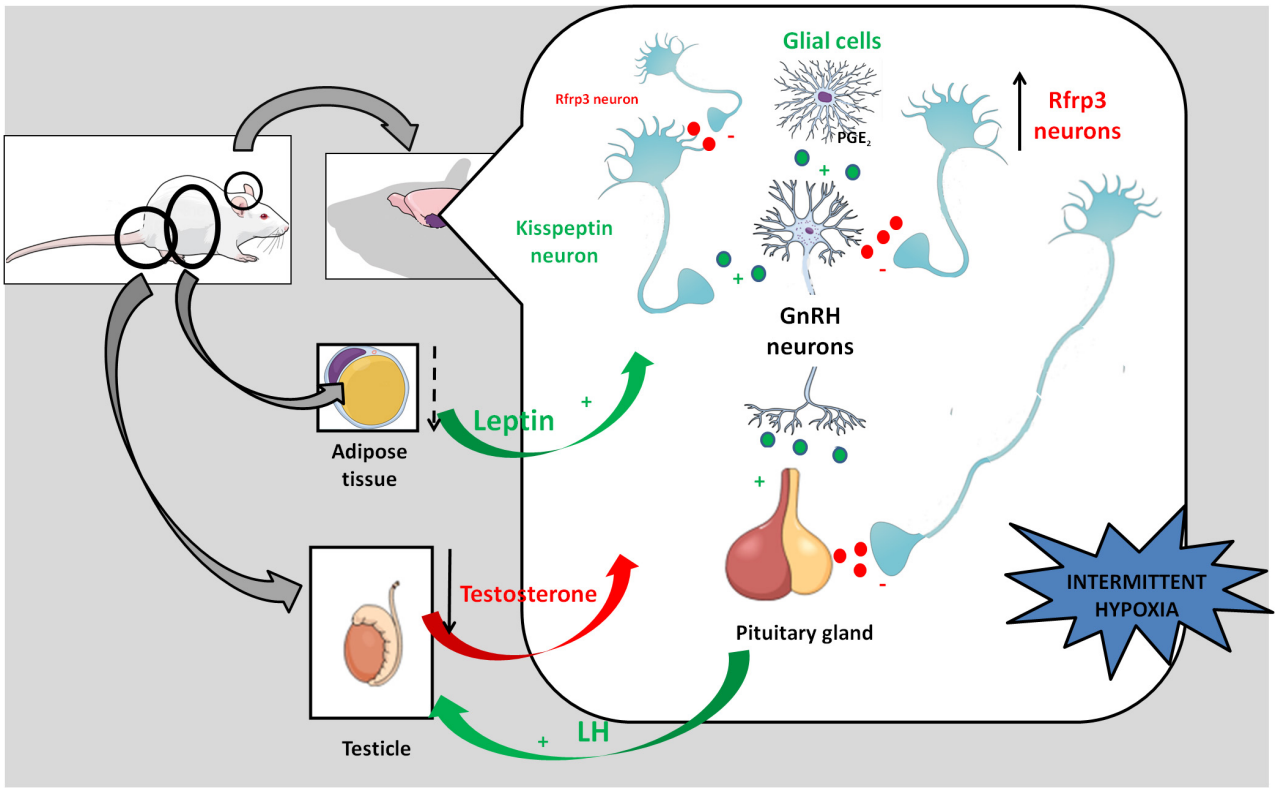

Fig. 4. Main findings and possible underlying mechanisms. Hypothalamus-pituitary-gonadal axis secretion is under the influence of multiple regulatory mechanisms, both central and non-central nervous system related stimuli. Using an in vivo animal model, it is demonstrated that exposure to chronic intermittent hypoxia decreases LH and testosterone concentration, as well as increases the levels of *Rfrp3* mRNA, a negative regulator for GnRH and LH secretion (principal findings are shown as full arrows). Additionally, animals exposed to CIH showed lower body weight, which may account for lower leptin (positive modulator for the hypothalamus-pituitary-gonadal axis) levels (hypothetic underlying mechanism, shown as dotted arrow). Direct effects of hypoxia on the testicles may contribute to the low testosterone concentration and should not be ruled out. Green indicates positive modulation for hormonal release, whereas red means negative modulation.

poxia [28]. Following a report by Walberg et al., that showed pituitary PGE_2 stimulates LH, PGE_2 in adenohypophysis tissue was also measured. As observed for hypothalamus tissue, no changes in astrocyte PGE_2 levels were found in any of the groups studied.

There are limitations to the present study that must be noted.t. Firstly, regulation of the HPG axis involves many more factors than those analyzed in the present work. Animal body weight, for example, is one such regulator, since body fat is directly associated with HPG activity. Leptin, an adipocyte-derived protein hormone, not only conveys a signal of the amount of energy stored to the central nervous system but also plays an important role in regulating neuroendocrine function [29]. It has been demonstrated that leptin stimulates GnRH and LH release [30]. Hence, the lower body weight observed in CIH animals suggests that leptin deficiency may also dysregulate GnRH and LH release in this experimental group. Further studies are needed to corroborate the role of body weight and leptin in the regulation of the hypothalamic-pituitary-gonadal axis. The main findings of this study and the possible underlying mechanisms discussed herein can be observed in Fig. 4. Secondly, only mRNA levels of *GhRH*, *Rfrp3* and *Kiss1* were assessed in the present study. It would be of interest to analyze protein expression of these molecules. Additionally, given that GnRH and LH are regulated by multiple factors, studies should be conducted to fully characterize the mechanisms mediating hypothalamic—pituitary response under conditions of chronic hypoxia.

In conclusion, intermittent hypoxia would seem to be more deleterious to male fertility than continuous hypoxia. This may be explained by the negative regulation of LH release by Rfrp3, which is overexpressed in the hypothalamus of CIH rats. Continuous exposure to hypoxia would enable acclimation mechanisms to maintain kisspeptin secretion, avoiding a decrease in LH levels. The observed alteration in HPG axis function may involve other mechanisms that were not analyzed in this study, such as impaired GnRH and LH release due to leptin stimulation. Direct effects of hypoxia on the testicles may contribute to the low testosterone concentration observed in intermittently exposed animals and should not be ruled out.

Abbreviations

AH, adenohypophysis; CCH, chronic continuous hypoxia; CIH, chronic intermittent hypoxia; FSH, follicle-stimulating hormone; GnRH, gonadotropin releasing hormone; HPG, hypothalamus-pituitary-gonadal axis; HX, hypoxia; LH, luteinizing hormone; OSA, obstructive sleep apnea; PGE2, prostaglandin E2; Rfrp-3, RFamide-related peptides; T, testosterone.

Author contributions

All authors participated in the conceptualization of the study, its methodological design and data analysis. ART performed the experiments, analyzed data and wrote the original draft. MPM and JFSJ were the project administrators and the responsible for grant acquisition. They also revised and edited the original draft.

Ethics approval and consent to participate

Animals were purchased from the animal housing facility of the School of Pharmacy and Biochemistry, University of Buenos Aires (Argentina). All the animals were treated in keeping with the NIH Guide for the Care and use of Laboratory animals (NIH 8th edition, 2011), and the protocols were approved by the institutional Animal Care and Use Committee (IACUC, N^o 11/06/2012-23) of the School of Dentistry, University of Buenos Aires.

Acknowledgment

Authors would like to thank Julia Astrauskas for her technical assistance.

Funding

This research was funded by UBACYT 20020150100006BA and PIP2015-2017 045 CONICET grants.

Conflict of interest

The authors declare no conflict of interest.

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