RESEARCH

The role of testosterone in the respiratory and thermal responses to hypoxia and hypercapnia in rats

Danuzia A Marques¹, Luis Gustavo A Patrone¹, Carolina S Scarpellini^{1,2}, Kênia C Bícago¹, Raphael E Szawka³ and Luciane H Gargaglioni¹

¹Department of Animal Morphology and Physiology, Faculty of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, São Paulo, Brazil

²Brain & Breathing Science Laboratory, Keenan Research Centre for Biomedical Science, St. Michael's Hospital, Toronto, Canada

³Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais – UFMG, Belo Horizonte, Minas Gerais, Brazil

Correspondence should be addressed to L H Gargaglioni: luciane.gargaglioni@unesp.br

Abstract

Many diseases of the respiratory system occur differently in males and females, indicating a possible role of gonadal hormones in respiratory control. We hypothesized that testosterone (T) is important for the ventilatory chemosensitivity responses in males. To test this hypothesis, we evaluated ventilation ($\dot{V}_E$), metabolic rate and body temperature (T_b) under normoxia/normocapnia, hypercapnia and hypoxia in orchietomized (ORX), ORX with testosterone replacement (ORX + T) or flutamide (FL, androgen receptor blocker)-treated rats. We also performed immunohistochemistry to evaluate the presence of androgen receptor (AR) in the carotid body (CB) of intact males. Orchietomy promoted a reduction $\dot{V}_E$ and ventilatory equivalent ($\dot{V}_E/\dot{V}O_2$) under room-air conditions, which was restored with testosterone treatment. Moreover, during hypoxia or hypercapnia, animals that received testosterone replacement had a higher $\dot{V}_E$ and $\dot{V}_E/\dot{V}O_2$ than control and ORX, without changes in metabolic and thermal variables. Flutamide decreased the hypoxic ventilatory response without changing the CO_2 -drive to breathe, suggesting that the testosterone effect on hypercapnic hyperventilation does not appear to involve the AR. We also determined the presence of AR in the CB of intact animals. Our findings demonstrate that testosterone seems to be important for maintaining resting $\dot{V}_E$ in males. In addition, the influence of testosterone on $\dot{V}_E$, either during resting conditions or under hypoxia and hypercapnia, seems to be a direct and specific effect, as no changes in metabolic rate or T_b were observed during any treatment. Finally, a putative site of testosterone action during hypoxia is the CB, since we detected the presence of AR in this structure.

Key Words

- ▶ gonadal hormone
- ▶ androgens
- ▶ breathing
- ▶ chemosensitivity

Journal of Endocrinology
(2020) **247**, 101–114

Introduction

Many breathing disorders, such as sleep apnea and sudden infant death syndrome (SIDS), have a higher occurrence in males than in females, which may indicate

the existence of sex differences in respiratory control (Kapsimalis & Kryger 2002, Chahrour & Zoghbi 2007, Behan & Wenninger 2008). A central issue that may play

an important role in the differences between males and females is the presence of sex hormones, for example, progesterone, estradiol and testosterone (T), produced by both sexes in different concentrations throughout life (Behan & Kinkead 2011). Many studies have suggested testosterone as a potential cause of the higher prevalence of sleep disorders in men, causing a destabilization in ventilatory control during sleep (Mateika *et al.* 2004, Lozo *et al.* 2017). This idea was reinforced by studies reporting that the apnea/hypopnea index increased in hypogonadal men after testosterone replacement (Matsumoto *et al.* 1985, Schneider *et al.* 1986). Further, inhibition of testosterone action via the 5 α -reductase pathway alleviates breathing instability during sleep (Chowdhuri *et al.* 2013). On the other hand, it has been shown that lower testosterone levels are correlated with chronic obstructive pulmonary disease (COPD) (Kaparianos *et al.* 2011) and anabolic steroid supplementation improved respiratory muscle strength and decreases hypoxemia (Schols *et al.* 1995). Furthermore, asthma prevails in boys before puberty and this sex preference reverses after puberty and in adulthood (Canguven & Albayrak 2011). Indeed, testosterone has a protective role in asthmatic patients, maintaining the physiological balance of autoimmunity and protective immunity by preserving the number of immune cells (Canguven & Albayrak 2011).

Data regarding the effect of testosterone on breathing control is still controversial, probably due to differences in experimental designs. For instance, some authors have shown that ventilation rises after testosterone replacement in both humans (White *et al.* 1985, Fogel *et al.* 2001) and animals (Tatsumi *et al.* 1994), whereas others found no change (Koepchen 1953, Tenorio-Lopes *et al.* 2017) or even inhibition of ventilation (Bayliss *et al.* 1987). In fact, it has been suggested that the impact of testosterone on breathing regulation in animals and humans requires its aromatization to estradiol. Thus, the role of testosterone in breathing function may ultimately be mediated by estradiol, the availability of which is controlled by aromatase (Simpson *et al.* 2002, Zabka *et al.* 2006). Likewise, there is no agreement regarding the role of testosterone in the ventilatory responses to hypercapnia and hypoxia (Matsumoto *et al.* 1985, White *et al.* 1985, Tatsumi *et al.* 1994, Joseph *et al.* 2002, Fournier *et al.* 2014, Boukari *et al.* 2016, Tenorio-Lopes *et al.* 2017).

Sex hormones receptors are widely distributed in brain regions important for respiratory control, such as the retrotrapezoid nucleus (RTN), nucleus of the solitary tract (NTS), locus coeruleus (LC), caudal medullary raphe and hypothalamus (Pascual *et al.* 2002,

Szawka *et al.* 2009, Behan & Kinkead 2011). Estradiol and progesterone receptors were also described in the carotid body (CB), a main peripheral chemosensitive site involved in the regulation of ventilation (Joseph *et al.* 2006). Although the presence of androgen receptor (AR) in brain chemosensitive sites is well known (Simerly *et al.* 1990), there are no data in the literature regarding the presence of AR in peripheral sites, such as the CB. A study by Tatsumi *et al.* (1994) demonstrated that testosterone replacement increased the carotid sinus nerve response to hypoxia, and this greater chemosensitivity was eliminated by the transection of the carotid sinus nerve. In contrast, there are no reports so far on the presence of AR in the cells of the CB.

Besides breathing control, testosterone can also regulate metabolic rate and body temperature (T_b). For instance, replacement of testosterone in hypogonadal men causes an increase in oxygen consumption ($\dot{V}O_2$) (White *et al.* 1985). Additionally, it was demonstrated that the pre-optic area of the hypothalamus (POA), an important region for T_b regulation, has a large expression of AR (Simerly *et al.* 1990) and testosterone affects the activity of temperature-sensitive and insensitive neurons in the POA (Silva & Boulant 1986). Thus, it is reasonable to consider a certain degree of interaction between metabolic, thermoregulatory and reproductive functions. Therefore, we further investigate the participation of testosterone and AR in the ventilatory, metabolic and thermal responses to hypoxia and hypercapnia in adult male rats.

Materials and methods

Animals

Experiments were performed on unanesthetized, adult male Wistar rats obtained from UNESP (Botucatu, Brazil; body mass, 250–310 g). The animals had free access to water and food, and were housed in a temperature-controlled room ($25 \pm 1^\circ\text{C}$) with a 12 h light:12 h darkness cycle (lights on at 06:30 h). The experimental protocols were run between 09:00 and 13:00 h. to avoid daily variations in hormone levels, and were in agreement with the guidelines of the National Council of Control in Animal Experimentation (CONCEA, Brazil) and approved by the local Animal Care and Use Committee (CEUA # 8.129/16).

Surgery

Animals were anesthetized with ketamine-xylazine (100 and 10 mg/kg, i.p., respectively) and the body area

designated for the procedure was shaved and cleaned with 2% chlorhexidine digluconate (Rioquímica, SP, Brazil). The animals were prophylactically treated with antibiotic (5% enrofloxacin, s.c.; 0.5 mg/kg, Schering-Plough) and, immediately after surgery, with analgesic anti-inflammatory (flunixin meglumine, i.m.; 2.5 mg/kg, Schering-Plough). Ten days before the experiments, the animals were submitted to the following surgical procedures.

Implantation of a temperature-measuring device

All animals were implanted with a miniature temperature datalogger (SubCue Dataloggers, Calgary, Canada) in the peritoneal cavity via midline laparotomy to record T_b . After implantation of the device, the abdominal muscles and skin were sutured in layers.

Orchiectomy, hormone and androgen receptor antagonist treatments

Three groups of rats (ORX, and ORX+T, ORX+FL) were submitted to bilateral orchiectomy by incision of the scrotum and ligation of the spermatic cords; the skin was then sutured. Three days after the surgeries, the animals were treated at 10:00 h daily for 7 consecutive days prior to the experiment with (1) vehicle (corn oil, ORX group; 0.2 mL/rat, s.c., Liza; Cargill, Sao Paulo, Brazil) or (2) testosterone propionate (ORX+T group; 0.25 mg/0.2 mL/rat, s.c.; dose: 830 µg/kg; Organon, Sao Paulo, Brazil). This dose has been reported to yield physiological testosterone levels in the plasma (Kalil *et al.* 2013). For controls, we used gonad-intact rats (intact group) and sham operation, which involved exposure of the testicles without excision (SHAM group). For the antagonist treatment, 3 days after the datalogger implantation, orchiectomized (ORX) or SHAM animals received the AR blocker flutamide (FL, 8 mg/kg/day, s.c.; Sigma) daily for 7 days prior to the experiment. The FL dose was determined according to previously published experiments (Reckelhoff *et al.* 1999, Davis *et al.* 2019).

After the experiments, animals were euthanized with tribromoethanol (0.3 mL/100 g, i.p.), the seminal vesicle was removed and its wet mass was measured. The mass of the seminal vesicle was used to confirm the effectiveness of the hormone treatment, as testosterone causes trophic effects on the seminal vesicle (de Carvalho *et al.* 2016).

Ventilatory, T_b , and metabolic measurements

Ventilation ($\dot{V}_E$) measurements were obtained using the barometric method (whole-body plethysmography),

as previously described by Drorbaugh & Fenn (1955) and performed in our laboratory (Biancardi *et al.* 2008, Marques *et al.* 2015).

Body temperature was recorded every 5 min by a temperature datalogger, as described in the 'Surgery' section. Metabolic rate was measured by indirect calorimetry ($\dot{V}O_2$) using a closed respirometry system, as described previously (Almeida *et al.* 2004, Marques *et al.* 2015). At the end of the normoxic/normocapnic, hypoxic and hypercapnic intervals, the airflow in the chamber was interrupted for 5 min, and the air was continuously sampled with an O_2 analyzer (PowerLab System, ADInstruments, Bella Vista, Australia). The percentage of oxygen decline inside the chamber was plotted against time, and the slope of the resulting curve was corrected for by the chamber volume and body mass of the animals in order to estimate the $\dot{V}O_2$ using the closed-circuit indirect calorimetry measures, as were used in our previous studies (Marques *et al.* 2015, 2017). $\dot{V}O_2$ was measured, and the ratio of carbon dioxide released to oxygen consumed ($\dot{V}CO_2/\dot{V}O_2$), called the respiratory quotient, was estimated in 0.85, as with previous studies in the literature (Raurich *et al.* 1989). The values are presented in mL of oxygen/min/kg in STPD (standard conditions of temperature, pressure, and dry air).

Before the experiments, the O_2 analyzer was calibrated with two known gas mixtures. Over the 5 min that the chamber was sealed, the reduction of the O_2 fraction inside was 0.5–1.0%, which is not considered significant to the animal (Marques *et al.* 2017). According to our previous measurements, the level of CO_2 inside the chamber at the end of a 5 min period with the rat breathing inside the box showed a small change (initial mean value of 0.033% CO_2 ; final mean value 0.2824% CO_2).

Experimental protocol

Each animal was individually placed inside a plethysmograph chamber (5 L), maintained at 25°C, and allowed to move while the chamber was flushed with humidified room air. The basal measurements (time zero) of $\dot{V}_E$ and $\dot{V}O_2$ were taken after the animal had acclimated for approximately 1 h. Subsequently, a hypercapnic gas mixture (7% CO_2 , 21% O_2 with N_2 balance; White Martins, Sao Paulo, Brazil) or a hypoxic mixture (7% O_2 with N_2 balance; White Martins) was flushed through the chamber for 30 min, and $\dot{V}_E$ and $\dot{V}O_2$ were measured at 30 min of exposure. The chamber was then flushed with room air for 90 min, so that the ventilation could return to basal values. After that, a hypoxic or hypercapnic gas mixture

was flushed into the chamber for 30 min, and $\dot{V}_E$ and $\dot{V}O_2$ were again measured at 30 min. The order of gas exposures (hypercapnic and hypoxic) was randomly chosen, and the CO_2 and O_2 percentages and exposure times were chosen based on pilot experiments and previous studies (Biancardi *et al.* 2008, Scarpellini *et al.* 2009).

CB removal and immunohistochemistry

We performed immunohistochemistry to verify the presence or absence of AR at the CB. This protocol was used for qualitative purposes only. Adult males were anesthetized with ketamine-xylazine (100 and 10 mg/kg, i.p., respectively), an incision in the ventral surface of the neck was made and, using forceps, the carotid bifurcations were removed *en bloc*. The dissected tissue was 24-h post-fixed in 4% PFA and maintained in a 30% sucrose solution at 4°C, as described by Joseph *et al.* (2006). The tissue was fixed in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA), cut into 40- μ m sections using a cryostat (CM1860 – Ag Protect; Leica), and mounted on glass slides for subsequent analysis.

Immunofluorescence

Glass slides containing CB sections were treated for 30 min in an antigenic retrieval solution (Dako) at 70°C. The slides were then cooled at room temperature for 20 min. Subsequently, the sections were washed with phosphate-buffered saline (PBS) five times for 5 min each, and then incubated for 1 h in a solution of PBS with 0.4% triton X-100 (TX-100) and 10% goat serum to prevent non-specific labeling, followed by 24 h of incubation with a mix of mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (1:400; T1299, Sigma-Aldrich) and rabbit anti-AR antibody (1:200; ab133273, Abcam) diluted in a solution of PBS with 0.4% TX-100 and 3% goat serum. On the next day, the slides were washed five times for 5 min each with PBS, and then incubated for 4 h at room temperature with a cocktail containing the AlexaFluor 594-coupled anti-mouse goat IgG and the AlexaFluor 488-coupled anti-rabbit goat IgG (1:1000; A11032 and A11015, Life Technologies) in PBS with 0.4% TX-100 and 3% goat serum. After 4 h, the slides were washed five times for 5 min in PBS, mounted with ProLong™ Gold (Molecular Probes) and sealed with a coverslip to be examined by confocal microscopy. We also included a negative (without primary antibody) control to test the specificity of the immunohistochemistry protocol

(Torlakovic *et al.* 2014). In addition, we simultaneously performed immunohistochemistry in the rat testis, a tissue well known to express AR-positive labeling (positive control) (Vornberger *et al.* 1994, Mayerhofer *et al.* 1996).

Statistical analyses

Data are presented as means $\pm$ S.E.M. Seminal vesicle mass, $\dot{V}_E$, f_R , V_T , T_b , $\dot{V}O_2$ and $\dot{V}_E/\dot{V}O_2$ data obtained during normoxia were analyzed using one-way ANOVA. The effects of treatments and gas exposure (hypoxia or hypercapnia) were analyzed using two-way ANOVA for repeated measures. Whenever ANOVA resulted in significant main effects or interactions, a Bonferroni post hoc test was performed to verify where the differences existed. Differences were considered to be significant when $P < 0.05$. Symbols (*) were used only when the post-test detected differences between treatments; P -values are shown in the graphs where pairs were analyzed.

Results

All data for ventilatory, metabolic and thermal variables tested during normoxic/normocapnic, hypoxic and hypercapnic gas exposures among groups are described in Tables 1 and 2. Two-way ANOVA values for the effects of treatments and gas exposure (hypoxia or hypercapnia) for all parameters of intact, ORX and ORX+T groups are described in Table 3. Table 4 presents the two-way ANOVA values for SHAM, SHAM+FL and ORX+FL animals. One unique aspect to this study was the use of a physiological dose of testosterone (a dose at which the resulting plasma concentration of testosterone following replacement was similar to levels found in intact rats) (Kalil *et al.* 2013), as well as the comparison of castrated with intact animals. No difference was observed between the intact and SHAM groups.

Effect of orchietomy, testosterone replacement and antagonist treatment on seminal vesicle mass

Orchietomy significantly reduced the seminal vesicle mass, compared to control animals (Table 1). On the other hand, testosterone replacement in ORX animals not only reversed the effect of removing the testicles, but caused a further increase in seminal vesicle mass (Table 1). Treatment with FL in SHAM rats caused a reduction in the seminal vesicle mass compared to the untreated SHAM group. ORX+FL rats displayed an even lower seminal

Table 1 Seminal vesicle mass, total ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f_R), oxygen consumption ($\dot{V}O_2$), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_b) data of intact, ORX (orchidectomized) and ORX + T (orchidectomized + testosterone) groups, during normoxic normocapnia (21% O_2 ; 0% CO_2), hypercapnia (7% CO_2) and hypoxia (7% O_2) exposure.

	INTACT ($n = 11$)	ORX ($n = 11$)	ORX + T ($n = 6$)
Seminal vesicle mass (mg/100 g)	336.6 $\pm$ 19.2	89.0 ^{a,b} $\pm$ 16.0	627.9 ^a $\pm$ 35.3
Normoxia			
$\dot{V}_E$ (mL/kg/min)	552.6 $\pm$ 23.8	471.5 ^{a,b} $\pm$ 19.1	612.1 $\pm$ 25.5
V_T (mL/kg)	6.2 $\pm$ 0.3	5.5 ^b $\pm$ 0.3	7.1 $\pm$ 0.4
f_R (breaths/min)	90.6 $\pm$ 3.4	86.0 $\pm$ 2.5	87.0 $\pm$ 5.4
$\dot{V}O_2$ (mL/kg/min)	18.0 $\pm$ 0.6	18.0 $\pm$ 0.6	19.7 $\pm$ 0.4
$\dot{V}_E/\dot{V}O_2$	30.7 $\pm$ 1.3	26.3 ^{a,b} $\pm$ 1.1	31.2 $\pm$ 1.6
T_b ($^{\circ}C$)	37.1 $\pm$ 0.1	37.0 $\pm$ 0.1	37.2 $\pm$ 0.1
Hypoxia			
$\dot{V}_E$ (mL/kg/min)	997.9 $\pm$ 54.6	950.0 $\pm$ 57.0	1341.8 ^a $\pm$ 109.5
V_T (mL/kg)	8.6 $\pm$ 0.1	8.7 $\pm$ 0.4	10.2 $\pm$ 0.6
f_R (breaths/min)	121.2 $\pm$ 5.7	109.0 $\pm$ 6.0	131.0 ^a $\pm$ 5.0
$\dot{V}O_2$ (mL/kg/min)	10.6 $\pm$ 0.6	11.5 $\pm$ 0.4	11.2 $\pm$ 0.3
$\dot{V}_E/\dot{V}O_2$	96.7 $\pm$ 5.8	85.1 ^b $\pm$ 4.8	119.4 ^a $\pm$ 7.4
T_b ($^{\circ}C$)	35.9 $\pm$ 0.1	35.9 $\pm$ 0.2	36.2 $\pm$ 0.3
Hypercapnia			
$\dot{V}_E$ (mL/kg/min)	1277.1 $\pm$ 46.1	1247.0 ^b $\pm$ 59.6	2002.1 ^a $\pm$ 188.7
V_T (mL/kg)	9.4 $\pm$ 0.3	9.4 $\pm$ 0.5	13.1 ^a $\pm$ 1.2
f_R (breaths/min)	136.7 $\pm$ 2.8	130.1 ^b $\pm$ 2.0	153.3 ^a $\pm$ 3.7
$\dot{V}O_2$ (mL/kg/min)	15.2 $\pm$ 0.4	15.7 $\pm$ 0.4	16.9 $\pm$ 0.4
$\dot{V}_E/\dot{V}O_2$	87.1 $\pm$ 2.9	78.9 ^b $\pm$ 4.0	124.3 ^a $\pm$ 8.8
T_b ($^{\circ}C$)	36.9 $\pm$ 0.1	37.0 $\pm$ 0.1	37.0 $\pm$ 0.2

Values are mean $\pm$ s.e.

^aSignificant difference from intact; ^bSignificant difference from ORX + T.

vesicle mass than SHAM+FL rats, further attesting to the antiandrogenic efficacy of FL (Table 2).

Effect of orchiectomy, testosterone replacement and antagonist treatment on thermal and metabolic responses to hypercapnia and hypoxia

Neither hypercapnia nor orchiectomy with or without testosterone replacement affected the T_b of rats. Regarding hypoxia, 7% O_2 decreased similarly T_b in all animals (Tables 1 and 2). The same response pattern was observed in animals that received FL treatment (Tables 3 and 4).

During room-air conditions, $\dot{V}O_2$ was not affected by orchiectomy, testosterone replacement or FL treatment (Tables 1 and 2). Hypoxia caused a reduction in $\dot{V}O_2$, with no difference among treatments (Tables 3 and 4). Hypercapnia affected $\dot{V}O_2$ in all groups with no differences among them (Tables 3 and 4).

Effect of orchiectomy, testosterone replacement and antagonist treatment on ventilation

Orchiectomy reduced $\dot{V}_E$ by 15% compared to the intact and ORX+T groups under room-air conditions,

mainly via an effect on V_T . Testosterone replacement restored the baseline $\dot{V}_E$ and V_T (Table 1). Flutamide treatment did not cause changes in $\dot{V}_E$ or V_T in either SHAM or ORX rats; however, FL promoted a decrease in the f_R of ORX+FL animals compared to the SHAM group (Table 2).

Hypoxia caused an increase in the ventilatory variables in all groups (Figs 1 and 2). Orchiectomy did not affect $\dot{V}_E$, V_T or f_R under hypoxia, compared to intact animals. On the other hand, testosterone replacement caused a further rise of $\dot{V}_E$, compared to the intact group, due to a higher f_R . Flutamide reduced the hypoxic ventilatory response in the SHAM group, compared to the untreated SHAM group, due to a decrease in f_R (Fig. 2).

Hypercapnia caused an increase in the ventilatory variables in all groups (Figs 3 and 4). CO_2 exposure increased all respiratory variables similarly in intact and ORX animals (Fig. 3). Nonetheless, a further increase in $\dot{V}_E$ and f_R was observed in ORX animals that received testosterone, compared to the intact and ORX groups (Fig. 3). Flutamide did not affect hypercapnic ventilatory response in either the ORX or SHAM groups (Fig. 4).

Regarding the air convection requirement ($\dot{V}_E/\dot{V}O_2$), Fig. 5 shows measurements of $\dot{V}_E/\dot{V}O_2$ for intact,

Table 2 Seminal vesicle mass, total ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f_R), oxygen consumption ($\dot{V}O_2$), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_b) of SHAM, SHAM + flutamide (SHAM + FL) and orchidectomized + flutamide (ORX + FL) groups, during normoxic normocapnia (21% O_2 ; 0% CO_2), hypercapnia (7% CO_2) and hypoxia (7% O_2) exposure.

	SHAM (n = 6)	SHAM+FL (n = 6)	ORX + FL (n = 6)
Seminal vesicle mass (mg/100 g)	322.4 ± 18.9	212.8 ^{a,b} ± 41.9	18.83 ^a ± 0.8
Normoxia			
$\dot{V}_E$ (mL/kg/min)	719.6 ± 25.2	727.4 ± 15.9	674.23 ± 34.5
V_T (mL/kg)	6.3 ± 0.2	6.7 ± 0.3	7.12 ± 0.2
f_R (breaths/min)	113.6 ± 3.9	109.5 ± 5.7	94.67 ^a ± 4.4
$\dot{V}O_2$ (mL/kg/min)	18.8 ± 0.7	17.4 ± 0.7	17.2 ± 1.0
$\dot{V}_E/\dot{V}O_2$	38.8 ± 1.8	42.1 ± 2.2	39.5 ± 1.8
T_b (°C)	37.2 ± 0.2	37.1 ± 0.1	36.93 ± 0.2
Hypoxia			
$\dot{V}_E$ (mL/kg/min)	1719.7 ± 77.8	1273.5 ^a ± 43.6	1341.5 ± 61.5
V_T (mL/kg)	9.2 ± 0.4	9.12 ± 0.26	10.0 ± 0.4
f_R (breaths/min)	188.0 ± 5.4	131.5 ^a ± 8.92	134.5 ± 4.4
$\dot{V}O_2$ (mL/kg/min)	16.8 ± 0.6	14.9 ± 1.2	14.8 ± 1.2
$\dot{V}_E/\dot{V}O_2$	102.6 ± 2.1	85.4 ^a ± 4.3	92.3 ± 6.0
T_b (°C)	36.4 ± 0.1	36.3 ± 0.2	36.5 ± 0.1
Hypercapnia			
$\dot{V}_E$ (mL/kg/min)	1875.9 ± 97.9	1908.7 ± 128.4	1939.9 ± 112.4
V_T (mL/kg)	12.4 ± 0.5	13.5 ± 0.6	14.8 ± 0.7
f_R (breaths/min)	151.0 ± 6.9	140.5 ± 5.2	131.5 ± 6.2
$\dot{V}O_2$ (mL/kg/min)	19.7 ± 0.9	20.1 ± 1.1	19.4 ± 0.9
$\dot{V}_E/\dot{V}O_2$	96.6 ± 7.8	96.4 ± 7.9	98.4 ± 5.5
T_b (°C)	37.2 ± 0.2	37.2 ± 0.1	37.0 ± 0.2

Values are mean ± S.E.

^aSignificant difference from SHAM; ^bSignificant difference from ORX + FL.

ORX and ORX with testosterone replacement. Orchiectomy decreased the $\dot{V}_E/\dot{V}O_2$ ratio under normoxia by 16%, and testosterone replacement restored it to baseline values (Fig. 5, top). Under hypoxia and hypercapnia, the $\dot{V}_E/\dot{V}O_2$ ratio

increased in all groups, with a further increase observed in ORX + T rats (Fig. 5, middle and bottom).

Flutamide treatment did not change $\dot{V}_E/\dot{V}O_2$ during normoxia or hypercapnia (Fig. 6, top and bottom).

Table 3 Two-way ANOVA results for total ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f_R), oxygen consumption ($\dot{V}O_2$), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_b) of intact, ORX (orchidectomized), and ORX + T (orchidectomized + testosterone) groups during hypoxia (7% O_2) and hypercapnia (7% CO_2) exposures compared with normoxic normocapnic condition.

	ANOVA results		
	Gas effect	Treatment effect	Factorial interaction
Hypoxia			
$\dot{V}_E$ (mL/kg/min)	$P < 0.0001$ $F_{(1,25)} = 241.70$	$P = 0.0005$ $F_{(2,25)} = 8.01$	$P = 0.04$ $F_{(2,25)} = 2.98$
V_T (mL/kg)	$P < 0.0001$ $F_{(1,25)} = 132.80$	$P = 0.06$ $F_{(2,25)} = 2.69$	NS
f_R (breaths/min)	$P < 0.0001$ $F_{(1,25)} = 68.28$	$P = 0.003$ $F_{(2,25)} = 5.59$	NS
$\dot{V}O_2$ (mL/kg/min)	$P < 0.0001$ $F_{(1,25)} = 578.90$	$P = 0.16$ $F_{(2,25)} = 1.99$	NS
$\dot{V}_E/\dot{V}O_2$	$P < 0.0001$ $F_{(1,25)} = 602.50$	$P = 0.0003$ $F_{(2,25)} = 12.14$	$P = 0.0002$ $F_{(2,25)} = 12.29$
T_b (°C)	$P < 0.0001$ $F_{(1,25)} = 102.00$	$P = 0.12$ $F_{(2,25)} = 2.078$	NS
Hypercapnia			
$\dot{V}_E$ (mL/kg/min)	$P < 0.0001$ $F_{(1,25)} = 523.30$	$P < 0.0001$ $F_{(2,25)} = 17.47$	$P < 0.0001$ $F_{(2,25)} = 12.24$
V_T (mL/kg)	$P < 0.0001$ $F_{(1,25)} = 271.70$	$P = 0.0007$ $F_{(2,25)} = 7.51$	$P = 0.002$ $F_{(2,25)} = 6.40$
f_R (breaths/min)	$P < 0.0001$ $F_{(1,25)} = 592.10$	$P = 0.004$ $F_{(2,25)} = 5.53$	$P = 0.0001$ $F_{(2,25)} = 9.91$
$\dot{V}O_2$ (mL/kg/min)	$P < 0.0001$ $F_{(1,25)} = 84.10$	$P = 0.08$ $F_{(2,25)} = 2.77$	NS
$\dot{V}_E/\dot{V}O_2$	$P < 0.0001$ $F_{(1,25)} = 439.70$	$P < 0.0001$ $F_{(2,25)} = 14.47$	$P = 0.0004$ $F_{(2,25)} = 10.73$
T_b (°C)	$P = 0.11$ $F_{(1,25)} = 2.75$	$P = 0.52$ $F_{(2,25)} = 0.76$	NS

Table 4 Two-way ANOVA results for total ventilation ($\dot{V}_E$), tidal volume (V_T), breathing frequency (f_R), oxygen consumption ($\dot{V}O_2$), respiratory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_b) data of SHAM, SHAM + flutamide (SHAM + FL) and orchidectomized + flutamide (ORX + FL) groups, during hypoxia (7% O_2) and hypercapnia (7% CO_2) exposures compared with normoxic normocapnic condition.

	ANOVA results		
	Gas effect	Treatment effect	Factorial interaction
Hypoxia			
$\dot{V}_E$ (mL/kg/min)	$P < 0.0001$ $F_{(1,15)} = 39.10$	$P = 0.00057$ $F_{(2,15)} = 12.27$	$P = 0.0006$ $F_{(2,15)} = 12.86$
V_T (mL/kg)	$P < 0.0001$ $F_{(1,15)} = 188.30$	$P = 0.092$ $F_{(2,15)} = 2.80$	NS
f_R (breaths/min)	$P < 0.0001$ $F_{(1,15)} = 103.00$	$P < 0.0001$ $F_{(2,15)} = 25.24$	$P = 0.0004$ $F_{(2,15)} = 13.83$
$\dot{V}O_2$ (mL/kg/min)	$P = 0.0007$ $F_{(1,15)} = 17.83$	$P = 0.25$ $F_{(2,15)} = 1.51$	NS
$\dot{V}_E/\dot{V}O_2$	$P < 0.0001$ $F_{(1,15)} = 511.70$	$P = 0.22$ $F_{(2,15)} = 1.65$	$P = 0.01$ $F_{(2,15)} = 6.28$
T_b ($^{\circ}C$)	$P < 0.0001$ $F_{(1,15)} = 69.10$	$P = 0.83$ $F_{(2,15)} = 0.19$	NS
Hypercapnia			
$\dot{V}_E$ (mL/kg/min)	$P < 0.0001$ $F_{(1,15)} = 461.70$	$P = 0.97$ $F_{(2,15)} = 0.023$	NS
V_T (mL/kg)	$P = 0.0001$ $F_{(1,15)} = 330.20$	$P = 0.0103$ $F_{(2,15)} = 6.30$	NS
f_R (breaths/min)	$P < 0.0001$ $F_{(1,15)} = 161.60$	$P = 0.044$ $F_{(2,15)} = 3.88$	NS
$\dot{V}O_2$ (mL/kg/min)	$P = 0.004$ $F_{(1,15)} = 11.52$	$P = 0.69$ $F_{(2,15)} = 0.37$	NS
$\dot{V}_E/\dot{V}O_2$	$P < 0.0001$ $F_{(1,15)} = 197.40$	$P = 0.95$ $F_{(2,15)} = 0.04$	NS
T_b ($^{\circ}C$)	$P = 0.77$ $F_{(1,15)} = 0.09$	$P = 0.52$ $F_{(2,15)} = 0.67$	NS

Figure 1

Effect of orchietomy and testosterone replacement on $\dot{V}_E$, V_T and f_R of rats exposed to hypoxia (7% O_2). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 1.

Figure 2

Effect of flutamide (FL) on $\dot{V}_E$, V_T and f_R of SHAM and orchietomized (ORX) rats exposed to hypoxia (7% O_2). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 2.

Figure 3

Effect of orchietomy and testosterone replacement on $\dot{V}_E$, $\dot{V}_T$ and f_R of rats exposed to hypercapnia (7% CO₂ and 21% O₂). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 1.

However, under hypoxia, the $\dot{V}_E/\dot{V}_{O_2}$ ratio was lower after FL treatment in the SHAM+FL group (Fig. 6, middle).

CB immunohistochemistry

Immunohistochemistry revealed that the CB (labeled for TH in red) is richly endowed with AR (labeled in green) (Fig. 7A, B and C). A negative (without primary antibody) labeling control can be seen in Fig. 7A and B, demonstrating the specificity of TH and AR immunoreactivity. At the same time, we performed the same double labeling in tissue from the rat testis, known to contain a high expression of AR, as a positive control, which further supports the specificity of AR labeling in the CB. This result is presented in Fig. 7D, E, F and G.

Discussion

The present study shows that orchietomy promoted a reduction in $\dot{V}_E$ and $\dot{V}_E/\dot{V}_{O_2}$ during room-air conditions,

Figure 4

Effect of flutamide (FL) on $\dot{V}_E$, $\dot{V}_T$ and f_R of SHAM and orchietomized (ORX) rats exposed to hypercapnia (7% CO₂ and 21% O₂). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 2.

and that testosterone replacement was able to restore it. Testosterone increased the ventilatory response to hypoxia and hypercapnia, without changing $\dot{V}_{O_2}$ and T_b , suggesting that this hormone may act directly on the peripheral and/or central chemoreflex. Treatment with the antiandrogenic agent FL reduces the hypoxia-induced hyperventilation, independently of metabolic changes, suggesting that testosterone provides an excitatory drive to breathe in hypoxic conditions. Accordingly, we demonstrate the presence of AR in the CB, indicating a putative site for testosterone action, which seems to be particularly relevant during hypoxia. The efficacy of orchietomy and testosterone replacement was confirmed by the respective effects in decreasing and increasing the mass of the seminal vesicles (de Carvalho *et al.* 2016). Flutamide treatment decreased the relative seminal vesicle weights in both SHAM and ORX rats, indicating the efficacy of the antiandrogenic effect in our model.

ORX rats experienced a decrease in ventilation during room-air conditions due to a lower $\dot{V}_T$, and testosterone replacement restored it to control values. These data are in contrast with previous studies, in which

Figure 5

Effect of orchietomy and testosterone replacement on respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) of rats exposed to normocapnia (0% CO_2 and 21% O_2 ; top), hypoxia (7% O_2 and 0% CO_2 ; middle) and hypercapnia (7% CO_2 and 21% O_2 ; bottom). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 1.

castration did not affect $\dot{V}_E$ (Bairam *et al.* 2009, Fournier *et al.* 2014, Tenorio-Lopes *et al.* 2017). The reason for this discrepancy might be related to the fact that we performed experiments 7 days after orchietomy, whereas these previous studies performed experiments 14 days after castration. Interestingly, FL treatment did not affect basal $\dot{V}_E$, suggesting that testosterone does act through AR to stimulate breathing under basal conditions. Because FL does not interfere with estrogenic actions or rapid androgen signaling, our present findings suggest that testosterone modulation of basal $\dot{V}_E$ may depend on the activation of membrane AR (Thomas 2019) or estrogen receptors, after aromatization to estradiol (Russell & Grossmann 2019). Thus, the diversity of cellular pathways through which testosterone may act adds further complexity to the understanding of its role in respiratory control.

It is known that elevated testosterone concentrations in the plasma have an impact on respiratory parameters (Fogel *et al.* 2001, Gonzales *et al.* 2011), and that AR is expressed in brainstem nuclei involved in breathing (Simerly *et al.* 1990). For instance, studies have demonstrated moderate labeling of AR in brain areas

Figure 6

Effect of flutamide (FL) on respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) of SHAM and orchietomized (ORX) rats exposed to normocapnia (0% CO_2 and 21% O_2 ; top), hypoxia (7% O_2 and 0% CO_2 ; middle) and hypercapnia (7% CO_2 and 21% O_2 ; bottom). Values are presented as % from baseline. *Significant statistical difference ($P < 0.05$) using one-way ANOVA with the Bonferroni post hoc test. Differences between pairs are represented and P -values are included when significant. In the graph, each point represents one animal's data; gross values are described in Table 2.

involved in the hypoxic and hypercapnic ventilatory response, such as the NTS, LC, parabrachial nucleus, periaqueductal gray (PAG) and C1 noradrenergic neurons (Hamson *et al.* 2004, Fournier *et al.* 2014). In fact, brainstem areas such as LC and ventrolateral PAG have the strongest AR staining compared to other brain regions (Hamson *et al.* 2004). Interestingly, LC neurons are considered central CO_2/H^+ chemoreceptors and modulate hypercapnic ventilatory response (Gargaglioni *et al.* 2010). In addition, a previous study of our laboratory showed that chemical lesion of the ventral PAG reduced the ventilatory response to hypercapnia, which demonstrates that this area also contributes to the CO_2 -drive to breathe (Lopes *et al.* 2012). Furthermore, AR is expressed not only in neurons, but also in glial cells, including astrocytes, oligodendrocytes and microglia, which play a key role in breathing control and chemosensitivity (Swift-Gallant *et al.* 2016). In fact, White *et al.* (1985) showed that $\dot{V}_E$ increases following testosterone administration in hypogonadal men, and that this increase is associated with comparable increases in metabolic rate. In women, testosterone also increases baseline ventilation during wakefulness (Fogel *et al.* 2001). On the other hand, Koepchen (1953) reported that administration of testosterone has no acute effects

Figure 7

Immunofluorescence for (A) tyrosine hydroxylase (TH-red) and (B) androgen receptor (AR-green) in the carotid body of an adult male rat. Photomicrographs are presented as positive staining in the left side and negative (without primary antibody) in the right side. (C) Detail of overlapping TH + AR photomicrograph. Immunohistology of the rat testis. (D) AR positive labeling in green. (E) Detail of AR labeling. (F) AR-negative photomicrograph (without primary antibody) with green background, and (G) detail of AR negative. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-20-0257>.

on ventilation in men under resting conditions, while in male cats, testosterone substantially inhibits phrenic activity (Bayliss *et al.* 1987).

In contrast to our previous studies in female rats, in which ovariectomy reduced the hypoxic and hypercapnic ventilatory response (Marques *et al.* 2015, 2017), no changes in the hypoxic and hypercapnic chemoreflexes were observed in ORX males in the present study (compared to intact rats). In addition, we show here that $\dot{V}O_2$ and T_b under hypercapnia or hypoxia were not affected by gonadectomy. More recently, Tenorio-Lopes *et al.* (2017) demonstrated that hyperventilation caused by 5% CO_2 exposure was not influenced by castration. However, at higher CO_2 levels (10%), ORX rats displayed a greater increase in the hypercapnic ventilatory response, compared to intact rats. Since we used 7% CO_2 , it is possible that higher levels of CO_2 are needed to observe

the castration effect on the response to hypercapnia; however, future experiments must be performed to verify this possibility.

According to the present study, supplementation with testosterone in ORX rats caused an increase in $\dot{V}_E$ under hypoxia and hypercapnia, without changing $\dot{V}O_2$, indicating a primary effect on respiratory chemosensitivity. The findings regarding the effect of testosterone treatment and the CO_2 -drive to breathe are contradictory. For instance, the hypercapnic ventilatory drive does not change significantly after testosterone treatment in hypogonadal men (Matsumoto *et al.* 1985, White *et al.* 1985). On the other hand, manipulation of testosterone levels in healthy women increases ventilatory sensitivity to CO_2 (Ahuja *et al.* 2007). The same was observed in castrated male cats, in which the administration of testosterone promoted a 47% increase

in the hypercapnic ventilatory response (Tatsumi *et al.* 1994). Additionally, testosterone has also been shown to depress the hypercapnic ventilatory drive in infant monkeys during sleep (Emery *et al.* 1994). In the present study, we used a paradigm of hormone treatment aimed to yield physiological levels of testosterone in the plasma (Kalil *et al.* 2013); therefore, the differential responses found in the literature may be related to the differences among species, experimental protocols, or even doses and regimens of testosterone treatment. Moreover, as observed for basal ventilation, FL treatment did not change the hypercapnic ventilatory response. This suggests that the effect of testosterone on the CO₂-drive to breath is not mediated by AR. As discussed previously, this is possibly another situation where testosterone effects may be due to its enzymatic conversion to estradiol (Samy *et al.* 2003) and/or through activation of nongenomic signaling mechanisms. Further studies are needed to clarify this issue.

We demonstrated that the hypoxic ventilatory response was also increased by testosterone replacement, as was observed in the study by Tatsumi *et al.* (1994), in which testosterone-treated male cats had a greater CB chemosensitivity to hypoxia. Regarding humans, one study found a decrease (Matsumoto *et al.* 1985), whereas another observed an increase (White *et al.* 1985) in the hypoxic ventilatory response after testosterone treatment in hypogonadal males. Our data showed that the androgen antagonist in SHAM rats decreased the hypoxic ventilatory response acting on breathing frequency and ventilatory equivalent ($\dot{V}_E/\dot{V}O_2$ - without changing $\dot{V}O_2$), suggesting a direct effect on hypoxic chemosensitivity. Furthermore, we performed immunohistochemistry to confirm the presence of AR in the CB, suggesting a cellular site for the effect of testosterone on hyperventilation during hypoxic conditions. Our findings provide evidence that testosterone leads to a higher $\dot{V}_E$ under low-O₂ partial pressure acting through AR that are, at least in part, located in peripheral chemoreceptors.

Regarding T_b, treatment with testosterone did not affect the changes induced by high levels of CO₂ or low levels of O₂. It is important to point out that, the effect of testosterone replacement was specific to the ventilatory measurements, without affecting thermal or metabolic variables, demonstrating a specific physiological effect.

Our study demonstrated that castrated animals that received testosterone had $\dot{V}_E$ increases of 57% under hypercapnia and 35% during hypoxia, compared to intact animals. Our study used adult, middle-aged animals that were gonad-intact, ORX or ORX+T. As far as we know,

previous studies that evaluated the effects of testosterone supplementation on ventilatory control in adult, middle-aged men or animals (the model for our study) did not use an intact group with normal physiological testosterone levels as a reference for comparison, which might have led them to underestimate the effects of testosterone on ventilation. Some studies evaluated ventilatory chemosensitivity changes in hypogonadal men with or without testosterone supplementation (Matsumoto *et al.* 1985, White *et al.* 1985, Schneider *et al.* 1986). Similarly, an animal study performed by Tatsumi *et al.* (1994) used castrated male cats with or without testosterone replacement. Thus, a unique factor in our study was the comparison of ORX animals with ORX+T replacement (a model similar to that found in the current literature) with gonad-intact subjects. The differences found in the present study, among others, demonstrated that the ORX+T group did not mimic the responses observed in the intact group, even when a physiological dose was used for supplementation, which sheds new light on the role of testosterone replacement in chemosensitivity. We also contributed more specific data to the literature about the specific action of testosterone using a specific androgen antagonist.

The different effects observed between endogenously produced testosterone and testosterone supplementation on ventilation might be related to the ultradian rhythm of testosterone release, or even because the testicles produce other hormones, such as anti-Müllerian hormone, inhibin, activin, and follistatin, among others (Ying 1988, Anderson *et al.* 1998, Matuszczak *et al.* 2013), which might also affect respiratory control. Therefore, future studies are needed to verify whether other testicular hormones are important for breathing control. Another possibility is that the testicles are also responsible for converting part of the testosterone produced into the potent metabolite, dihydrotestosterone (DHT) (Folman *et al.* 1972, Dorrington & Fritz 1975, Steers 2001, Liu & Veldhuis 2018). Thus, the role of DHT in ventilation remains unknown.

Androgens, such as testosterone, are used by people with few restrictions (Behan & Kinkead 2011). In our work, we demonstrated that testosterone can change ventilatory chemosensitivity. In 2017, the Forum of International Respiratory Societies (2017) published a report which asserts that more than one billion people in the world suffer from acute or chronic respiratory diseases. Interestingly, men are more likely than women to be affected, which brings us to the discussion at hand, where we highlight the role of sex hormones in the control of the respiratory drive (for a review, see Gargaglioni *et al.* 2019). Here we suggest that

androgenic treatments, as well as the development of new synthetic androgenic drugs, should take into account the impacts they can have on the respiratory system in men.

In summary, the present study demonstrated that orchietomy decreases ventilation in rats during room-air conditions, and that testosterone is able to restore it. In addition, testosterone replacement promotes an increase in the ventilatory chemosensitivity under hypoxia without changing metabolism or T_b , suggesting that androgens stimulate the respiratory response to low- O_2 levels. A putative site of testosterone action is the CB, since we detected the presence of AR in this structure. Nevertheless, testosterone could also be acting on the CNS, since AR is present in respiratory regions. Testosterone administration also increased the hypercapnic ventilatory response; however, FL did not affect the CO_2 -drive to breathe, suggesting that the testosterone effect does not appear to involve the AR. The present findings contribute to elucidate the modulation of androgens on the control of ventilation, metabolism, and T_b adult male rats.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work received financial support from the Sao Paulo Research Foundation (FAPESP; 2019/09469-8) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – 407490/2018-3). D A M was the recipient of a FAPESP scholarship 2016/04276-9 and CNPq 140715/2015-0.

Acknowledgements

We thank Dr Vincent Joseph for the kind donation of the anti-AR antibody and discussions, Dr Richard Kinkead for the suggestions and Dr Elaine da Silva for CB removal assistance.

References

- Ahuja D, Mateika JH, Diamond MP & Safwan Badr MS 2007 Ventilatory sensitivity to carbon dioxide before and after episodic hypoxia in women treated with testosterone. *Journal of Applied Physiology* **102** 1832–1838. (<https://doi.org/10.1152/japplphysiol.01178.2006>)
- Almeida MC, Steiner AA, Coimbra NC & Branco LG 2004 Thermoeffector neuronal pathways in fever: a study in rats showing a new role of the locus coeruleus. *Journal of Physiology* **558** 283–294. (<https://doi.org/10.1113/jphysiol.2004.066654>)
- Anderson RA, Evans LW, Irvine DS, McIntyre MA, Groome NP & Riley SC 1998 Follistatin and activin A production by the male reproductive tract. *Human Reproduction* **13** 3319–3325. (<https://doi.org/10.1093/humrep/13.12.3319>)
- Bairam A, Montandon G, Joseph V, Lajeunesse Y & Kinkead R 2009 Enhancement of the breathing frequency response to hypoxia by neonatal caffeine treatment in adult male rats: the role of testosterone. *Respiratory Physiology and Neurobiology* **165** 261–265. (<https://doi.org/10.1016/j.res.2008.11.002>)
- Bayliss DA, Millhorn DE, Gallman EA & Cidlowski JA 1987 Progesterone stimulates respiration through a central nervous system steroid receptor-mediated mechanism in cat. *PNAS* **84** 7788–7792. (<https://doi.org/10.1073/pnas.84.21.7788>)
- Behan M & Kinkead R 2011 Neuronal control of breathing: sex and stress hormones. *Comprehensive Physiology* **1** 2101–2139. (<https://doi.org/10.1002/cphy.c100027>)
- Behan M & Wenninger JM 2008 Sex steroidal hormones and respiratory control. *Respiratory Physiology and Neurobiology* **164** 213–221. (<https://doi.org/10.1016/j.res.2008.06.006>)
- Biancardi V, Bicego KC, Almeida MC & Gargaglioni LH 2008 Locus coeruleus noradrenergic neurons and CO_2 drive to breathing. *Pflugers Archiv* **455** 1119–1128. (<https://doi.org/10.1007/s00424-007-0338-8>)
- Boukari R, Rossignol O, Marcouiller F, Bairam A & Joseph V 2016 Membrane progesterone receptors α and β contribute to regulation of breathing in adult male and female mice. *American Thoracic Society* **201** A2558. (<https://doi.org/10.1152/jappphysiol.00397.2016>)
- Canguven O & Albayrak S 2011 Do low testosterone levels contribute to the pathogenesis of asthma? *Medical Hypotheses* **76** 585–588. (<https://doi.org/10.1016/j.mehy.2011.01.006>)
- Chahrour M & Zoghbi HY 2007 The story of Rett syndrome: from clinic to neurobiology. *Neuron* **56** 422–437. (<https://doi.org/10.1016/j.neuron.2007.10.001>)
- Chowdhuri S, Bascom A, Mohan D, Diamond MP & Badr MS 2013 Testosterone conversion blockade increases breathing stability in healthy men during NREM sleep. *Sleep* **36** 1793–1798. (<https://doi.org/10.5665/sleep.3202>)
- Davis GK, Intapad S, Newsome AD, Coats LE, Bamrick DR, Ojeda NB & Alexander BT 2019 Androgen receptor blockade differentially regulates blood pressure in growth-restricted versus ovarian deficient rats. *Hypertension* **74** 975–982. (<https://doi.org/10.1161/HYPERTENSIONAHA.119.13257>)
- de Carvalho D, Marques DAAD, Bernuci MPP, Leite CMM, Araújo-Lopes R, Anselmo-Franci J, Bicego KCC, Szawka REE & Gargaglioni LHH 2016 Role of sex hormones in hypercapnia-induced activation of the locus coeruleus in female and male rats. *Neuroscience* **313** 36–45. (<https://doi.org/10.1016/j.neuroscience.2015.11.026>)
- Dorrington JH & Fritz IB 1975 Cellular localization of 5α -reductase and 3α -hydroxysteroid dehydrogenase in the seminiferous tubule of the rat testis. *Endocrinology* **96** 879–889. (<https://doi.org/10.1210/endo-96.4.879>)
- Drorbaugh JE & Fenn WO 1955 A barometric method for measuring ventilation in newborn infants. *Pediatrics* **16** 81–87.
- Emery MJ, Hlastala MP & Matsumoto AM 1994 Depression of hypercapnic ventilatory drive by testosterone in the sleeping infant primate. *Journal of Applied Physiology* **76** 1786–1793. (<https://doi.org/10.1152/jappl.1994.76.4.1786>)
- Fogel RB, Malhotra A, Pillar G, Pittman SD, Dunaif A & White DP 2001 Increased prevalence of obstructive sleep apnea syndrome in obese women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* **86** 1175–1180. (<https://doi.org/10.1210/jcem.86.3.7316>)
- Folman Y, Haltmeyer GC & Eik-Nes KB 1972 Production and secretion of 5 α dihydrotestosterone by the dog testis. *American Journal of Physiology* **222** 653–656. (<https://doi.org/10.1152/ajplegacy.1972.222.3.653>)
- Forum of International Respiratory Societies 2017 The global impact of respiratory disease – second edition. Sheffield, UK: European Respiratory Society. (available at: https://www.who.int/gard/publications/The_Global_Impact_of_Respiratory_Disease.pdf)

- Fournier S, Gulemetova R, Joseph V & Kinkad R 2014 Testosterone potentiates the hypoxic ventilatory response of adult male rats subjected to neonatal stress. *Experimental Physiology* **99** 824–834. (<https://doi.org/10.1113/expphysiol.2013.077073>)
- Gargaglioni LH, Hartzler LK & Putnam RW 2010 The locus coeruleus and central chemosensitivity. *Respiratory Physiology & Neurobiology* **173** 264–273. (<https://doi.org/10.1016/j.resp.2010.04.024>)
- Gargaglioni LH, Marques DA & Patrone LGA 2019 Sex differences in breathing. *Comparative Biochemistry and Physiology: Part A, Molecular and Integrative Physiology* **238** 110543. (<https://doi.org/10.1016/j.cbpa.2019.110543>)
- Gonzales GF, Tapia V, Gasco M & Gonzales-Castañeda C 2011 Serum testosterone levels and score of chronic mountain sickness in Peruvian men natives at 4340m. *Andrologia* **43** 189–195. (<https://doi.org/10.1111/j.1439-0272.2010.01046.x>)
- Hamson DK, Jones BA & Watson NV 2004 Distribution of androgen receptor immunoreactivity in the brainstem of male rats. *Neuroscience* **127** 797–803. (<https://doi.org/10.1016/j.neuroscience.2004.06.006>)
- Joseph V, Soliz J, Soria R, Pequignot J, Favier R, Spielvogel H & Pequignot JM 2002 Dopaminergic metabolism in carotid bodies and high-altitude acclimatization in female rats. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **282** R765–R773. (<https://doi.org/10.1152/ajpregu.00398.2001>)
- Joseph V, Doan VD, Morency C-EE, Lajeunesse Y & Bairam A 2006 Expression of sex-steroid receptors and steroidogenic enzymes in the carotid body of adult and newborn male rats. *Brain Research* **1073–1074** 71–82. (<https://doi.org/10.1016/j.brainres.2005.12.075>)
- Kalil B, Leite CM, Carvalho-Lima M & Anselmo-Franci JA 2013 Role of sex steroids in progesterone and corticosterone response to acute restraint stress in rats: sex differences. *Stress* **16** 452–460. (<https://doi.org/10.3109/10253890.2013.777832>)
- Kaparianos A, Argyropoulou E, Efremidis G & Spiropoulos K 2011 Sex hormone alterations and systemic inflammation in a group of male COPD smokers and their correlation with the +138 insA/delA endothelin-1 gene polymorphism. A case-control study. *European Review for Medical and Pharmacological Sciences* **15** 1149–1157.
- Kapsimalis F & Kryger MH 2002 Gender and obstructive sleep apnea syndrome, Part 1: clinical features. *Sleep* **25** 412–419. (<https://doi.org/10.1093/sleep/25.4.409>)
- Koepchen HP 1953 Effect of cortisone and testosterone on respiration. *Pflügers Archiv* **257** 144–154. (<https://doi.org/10.1007/BF00412468>)
- Liu PY & Veldhuis JD 2018 Hypothalamo-pituitary unit, testis, and male accessory organs. In *Yen and Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*, 8th ed., pp. 285–300.e8. Amsterdam, Netherlands: Elsevier. (<https://doi.org/10.1016/B978-0-323-47912-7.00012-3>)
- Lopes LT, Patrone LG, Bicego KC, Coimbra NC & Gargaglioni LH 2012 Periaqueductal gray matter modulates the hypercapnic ventilatory response. *Pflügers Archiv* **464** 155–166 (<https://doi.org/10.1007/s00424-012-1119-6>)
- Lozo T, Komnenov D, Badr MS & Mateika JH 2017 Sex differences in sleep disordered breathing in adults. *Respiratory Physiology and Neurobiology* **245** 65–75. (<https://doi.org/10.1016/j.resp.2016.11.001>)
- Marques DA, de Carvalho D, da Silva GSF, Szawka RE, Anselmo-Franci JA, Bicego KC & Gargaglioni LH 2015 Ventilatory, metabolic, and thermal responses to hypercapnia in female rats: effects of estrous cycle, ovariectomy, and hormonal replacement. *Journal of Applied Physiology* **119** 61–68. (<https://doi.org/10.1152/japplphysiol.00254.2015>)
- Marques DA, de Carvalho D, da Silva GSF, Szawka RE, Anselmo-Franci JA, Bicego KC & Gargaglioni LH 2017 Influence of estrous cycle hormonal fluctuations and gonadal hormones on the ventilatory response to hypoxia in female rats. *Pflügers Archiv* **469** 1277–1286. (<https://doi.org/10.1007/s00424-017-2022-y>)
- Mateika JH, Mendello C, Obeid D & Badr MS 2004 Peripheral chemoreflex responsiveness is increased at elevated levels of carbon dioxide after episodic hypoxia in awake humans. *Journal of Applied Physiology* **96** 1197–1205; discussion 1196. (<https://doi.org/10.1152/japplphysiol.00573.2003>)
- Matsumoto AM, Sandblom RE, Schoene RB, Lee KA, Giblin EC, Pierson DJ & Bremner WJ 1985 Testosterone replacement in hypogonadal men: effects on obstructive sleep apnoea, respiratory drives, and sleep. *Clinical Endocrinology* **22** 713–721. (<https://doi.org/10.1111/j.1365-2265.1985.tb00161.x>)
- Matuszczak E, Hermanowicz A, Komarowska M & Debek W 2013 Serum AMH in physiology and pathology of male gonads. *International Journal of Endocrinology* **2013** 128907. (<https://doi.org/10.1155/2013/128907>)
- Mayerhofer A, Danilchik M, Pau KY, Lara HE, Russell LD & Ojeda SR 1996 Testis of prepubertal rhesus monkeys receives a dual catecholaminergic input provided by the extrinsic innervation and an intragonadal source of catecholamines. *Biology of Reproduction* **55** 509–518. (<https://doi.org/10.1095/biolreprod55.3.509>)
- Pascual O, Morin-Surun MP, Barna B, Denavit-Saubié M, Pequignot JM & Champagnat J 2002 Progesterone reverses the neuronal responses to hypoxia in rat nucleus tractus solitarius in vitro. *Journal of Physiology* **544** 511–520. (<https://doi.org/10.1113/jphysiol.2002.023994>)
- Raurich JM, Ibañez J & Marse P 1989 Validation of a new closed circuit indirect calorimetry method compared with the open Douglas bag method. *Intensive Care Medicine* **15** 274–278. (<https://doi.org/10.1007/BF00271067>)
- Reckelhoff JF, Zhang H, Srivastava K & Granger JP 1999 Androgens and androgen receptor gender differences in hypertension in spontaneously hypertensive rats: role of. *Hypertension* **34** 920–923. (<https://doi.org/10.1161/01.HYP.34.4.920>)
- Russell N & Grossmann M 2019 MECHANISMS IN ENDOCRINOLOGY: Estradiol as a male hormone. *European Journal of Endocrinology* **181** R23–R43. (<https://doi.org/10.1530/EJE-18-1000>)
- Samy TSA, Zheng R, Matsutani T, Rue LW, Bland KI & Chaudry IH 2003 Mechanism for normal splenic T lymphocyte functions in proestrus females after trauma: enhanced local synthesis of 17 β -estradiol. *American Journal of Physiology: Cell Physiology* **285** C139–C149. (<https://doi.org/10.1152/ajpcell.00058.2003>)
- Scarpellini C da S, Gargaglioni LH, Branco LGS & Bicego KC 2009 Role of preoptic opioid receptors in the body temperature reduction during hypoxia. *Brain Research* **1286** 66–74. (<https://doi.org/10.1016/j.brainres.2009.06.039>)
- Schneider BK, Pickett CK, Zwillich CW, Weil JV, McDermott MT, Santen RJ, Varano LA & White DP 1986 Influence of testosterone on breathing during sleep. *Journal of Applied Physiology* **61** 618–623. (<https://doi.org/10.1152/jappl.1986.61.2.618>)
- Schols AM, Soeters PB, Mostert R, Pluymers RJ & Wouters EF 1995 Physiologic effects of nutritional support and anabolic steroids in patients with chronic obstructive pulmonary disease. A placebo-controlled randomized trial. *American Journal of Respiratory and Critical Care Medicine* **152** 1268–1274. (<https://doi.org/10.1164/ajrccm.152.4.7551381>)
- Silva NL & Boulant JA 1986 Effects of testosterone, estradiol, and temperature on neurons in preoptic tissue slices. *American Journal of Physiology* **250** R625–R632. (<https://doi.org/10.1152/ajpregu.1986.250.4.R625>)
- Simerly RB, Chang C, Muramatsu M & Swanson LW 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. *Journal of Comparative Neurology* **294** 76–95. (<https://doi.org/10.1002/cne.902940107>)
- Simpson ER, Clyne C, Rubin G, Boon B, Robertson K, Britt K, Speed C & Jones M 2002 Aromatase – a brief overview. *Annual Review of Physiology* **64** 93–127. (<https://doi.org/10.1146/annurev.physiol.64.081601.142703>)
- Steers WD 2001 5 α -Reductase activity in the prostate. *Urology* **58** (Supplement 1) 17–24; discussion 24. ([https://doi.org/10.1016/S0090-4295\(01\)01299-7](https://doi.org/10.1016/S0090-4295(01)01299-7))

- Swift-Gallant A, Coome LA, Ramzan F & Monks DA 2016 Nonneural androgen receptors affect sexual differentiation of brain and behavior. *Endocrinology* **157** 788–798. (<https://doi.org/10.1210/en.2015-1355>)
- Szawka RE, Rodovalho GV, Monteiro PM, Carrer HF & Anselmo-Franci JA 2009 Ovarian-steroid modulation of locus coeruleus activity in female rats: involvement in luteinising hormone regulation. *Journal of Neuroendocrinology* **21** 629–639. (<https://doi.org/10.1111/j.1365-2826.2009.01880.x>)
- Tatsumi K, Hannhart B, Pickett CK, Weil JV. & Moore LG 1994 Effects of testosterone on hypoxic ventilatory and carotid body neural responsiveness. *American Journal of Respiratory and Critical Care Medicine* **149** 1248–1253. (<https://doi.org/10.1164/ajrccm.149.5.8173766>)
- Tenorio-Lopes L, Henry MS, Marques D, Tremblay MÈ, Drolet G, Bretzner F & Kinkead R 2017 Neonatal maternal separation opposes the facilitatory effect of castration on the respiratory response to hypercapnia of the adult male rat: evidence for the involvement of the medial amygdala. *Journal of Neuroendocrinology* **29** e12550. (<https://doi.org/10.1111/jne.12550>)
- Thomas P 2019 Membrane androgen receptors unrelated to nuclear steroid receptors. *Endocrinology* **160** 772–781. (<https://doi.org/10.1210/en.2018-00987>)
- Torlakovic EE, Francis G, Garratt J, Gilks B, Hyjek E, Ibrahim M, Miller R, Nielsen S, Petcu EB, Swanson PE, *et al.* 2014 Standardization of negative controls in diagnostic immunohistochemistry: recommendations from the international ad hoc expert panel. *Applied Immunohistochemistry and Molecular Morphology* **22** 241–252. (<https://doi.org/10.1097/PAI.000000000000069>)
- Vornberger W, Prins G, Musto NA & Suarez-Quian CA 1994 Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* **134** 2307–2316. (<https://doi.org/10.1210/endo.134.5.8156934>)
- White DP, Schneider BK, Santen RJ, McDermott M, Pickett CK, Zwillich CW & Weil JV 1985 Influence of testosterone on ventilation and chemosensitivity in male subjects. *Journal of Applied Physiology* **59** 1452–1457. (<https://doi.org/10.1152/jappl.1985.59.5.1452>)
- Ying SY 1988 Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews* **9** 267–293. (<https://doi.org/10.1210/edrv-9-2-267>)
- Zabka AG, Mitchell GS & Behan M 2006 Conversion from testosterone to oestradiol is required to modulate respiratory long-term facilitation in male rats. *Journal of Physiology* **576** 903–912. (<https://doi.org/10.1113/jphysiol.2006.114850>)

Received in final form 21 July 2020

Accepted 4 August 2020

Accepted Manuscript published online 4 August 2020