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Review Article

Maternal thyroid hormone deficiency and cardiorespiratory disorder in rat pups

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ABSTRACT

During gestation, the mother is the main source of thyroid hormones for the foetus. Thus, hypothyroidism during pregnancy and/or preterm birth compromise thyroid hormone supply for the foetus. Maternal hypothyroidism increases risk of preterm birth and both conditions are associated with respiratory distress in infants. Since thyroid hormones are essential for normal brain development, it is plausible that maternal thyroid hormone deficiency plays a role in respiratory disorders related to neurological immaturity in the newborn; however, this hypothesis is yet to be tested. Here, we used methimazole treatment (MMI; 0.05% v/w) from the onset of pregnancy until two weeks postpartum to induce thyroid hormone deficiency in rat pups. At 14–15 days of age, we used plethysmography to measure breathing at rest and in response to hypoxia (12% O_2, 20 min) in intact pups. We then used a urethane/chloralose anesthetised preparation to measure cardiorespiratory inhibition induced by laryngeal chemoreflex stimulation. In intact pups, basal breathing did not differ between groups but the breathing frequency response to hypoxia of MMI-treated pups was lower than controls. Following anesthesia, breathing frequency of MMI pups was 60% lower than controls; following laryngeal chemoreflex stimulation, the drop in O_2 saturation that was 82% greater in MMI-treated pups than controls. Inactivation of $GABA_A$ receptors (bicuculline; 0.5 mg/kg) raised the frequency of anesthetised MMI pups but not control. We conclude that gestational thyroid hormone deficiency interferes with the respiratory and autonomic control systems of the offspring. Thyroid hormone supplementation could alleviate cardiorespiratory disorders in newborn, especially those born preterm.

1. Introduction

Thyroxine (T4; 3.5.3′0.5′-tetraiodothyronine) and triiodothyronine (T3; 3.5.3′-triiodothyronine) are the most important thyroid hormones. T4 is the main hormone produced by the thyroid gland. Upon its release in the circulation, T4 binds to transport proteins (thyroxin-binding globulin TBG; transthyretin TTR or albumin) that ensure delivery to various tissues, including the central nervous system (CNS)(Moog et al., 2017). Once within the CNS, glial cells that contain iodothyronine deiodinase (D2) convert T4 into its bioactive form (T3); it is estimated that ~80% of T3 within the CNS results from this conversion process (Crantz et al., 1982; Mohacsik et al., 2011).

During the perinatal period, thyroid hormones regulate many aspects of CNS development including neurogenesis, cell migration, dendrite and axon outgrowth, synapse formation, and myelination (Koibuchi and Chin, 2000; Oppenheimer and Schwartz, 1997). It is during these early life stages that the CNS is most vulnerable to thyroid hormone deficiency. In the foetus, thyroid gland function does not start before embryonic day 17.5–18 in rats and only during the second trimester in humans (Bernal, 2007); thus during early gestation, the mother is the unique source of thyroid hormones for the foetus.

Hypothyroidism is one of the most common diseases in pregnancy with a prevalence ranging from 0.3% to 4% of women (Baldy et al., 2016; Shinohara et al., 2018). Besides its impact on maternal health, hypothyroidism increases the risk of preterm birth and low birth weight in the infant (Baldy et al., 2016; Shinohara et al., 2018). Thus depending on timing, premature birth can compound this problem by causing a precocious interruption of maternal hormone supply and interfering with the postnatal surge of thyroid hormones (Biswas et al., 2002). As a result, thyroid hormones levels of infants born prior to 30 weeks of gestation are 57% lower than those born at term, a condition that increases the risk of neurodevelopmental delays (Lain et al., 2016).

In neonatal intensive care units, respiratory disorders related to

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immaturity of the respiratory control system is an important concern for clinicians (Eichenwald et al., 2016; Martin and Wilson, 2012). Preterm infants often show recurrent apneas with significant and potentially life threatening arterial O_2 desaturations and bradycardias. In these infants, a reduced responsiveness to respiratory stimuli (O_2 and CO_2) contributes to this respiratory disorder. Immaturity also compromises the ability to coordinate breathing with other functions such as swallowing (Martin and Wilson, 2012). The laryngeal chemoreflex is a set of responses aiming to prevent aspiration of foreign substances into the airways (Reix et al., 2007) and in a mature mammal, the presence of foreign substances (mostly liquids) near the larynx generally triggers coughing and swallowing with brief interruptions of breathing. In preterm infants, however, laryngeal chemoreflex stimulation leads to cessation of breathing movements with significant bradycardias, laryngospasm, systemic hypertension, and blood flow redistribution (Rousseau et al., 2017; Thach, 2001). Excessive laryngeal chemoreflex-induced cardiorespiratory depression is a significant concern for clinicians since in extreme cases these events can be fatal. As a result, delayed or abnormal laryngeal chemoreflex maturation has been linked with sudden infant death syndrome (SIDS) (Leiter and Bohm, 2007; Praud, 2010).

Maternal hypothyroidism has been associated with increased rates of respiratory distress syndrome in newborn (Biswas et al., 2002; Casey et al., 2005; Männistö et al., 2013). Furthermore, the severity of respiratory illness measured in preterm infants at birth is inversely proportional to the level of free T4 (Paul et al., 2010). Together, these observations suggest that withdrawing the foetus from the maternal source of thyroid hormones contributes to respiratory disorders related to immaturity of the neural control network. To the best of our knowledge, however, this hypothesis is yet to be addressed experimentally. Here, we used maternal methimazole treatment (MMI), an established experimental protocol that reliably induces thyroid hormone deficiency during gestation and the days following birth (Ahmed et al., 2010; Ahmed et al., 2012; Darbra et al., 2004). The consequences of MMI treatment on respiratory control was first tested in awake, unrestrained pups at rest and in response to a moderate hypoxic challenge. We then used an anesthetised pup preparation to compare the functionality of the laryngeal chemoreflex between groups. Based on the pronounced inhibitory responses observed in thyroid hormone deficient pups, we then used pharmacological treatment to determine whether MMI potentiates GABAergic modulation of respiratory activity in rat pups.

2. Methods

2.1. Ethical approval

Université Laval Animal Care Committee approved all the experimental procedures and protocols (CPAC protocol #2015030) which were in accordance with the guidelines of the Canadian Council on Animal Care and the ARRIVE guidelines. Experiments were performed on 62 Sprague-Dawley rat pups of both sexes aged of 14 to 15 days old. All animals used in this study were born and raised in our animal care facilities. All animals were maintained in standard laboratory conditions (21 °C, 12:12-h dark-light cycle: lights on at 6:00 and off at 18:00); the access to food and water was *ad libitum* during gestation and during experimental treatment. Distinct groups of animals were used for plethysmographic recordings and laryngeal chemoreflex stimulation protocol.

2.2. Maternal treatment and thyroid hormone deficiency in rat pups

Adult nulliparous female rats were randomly assigned to one of two groups: thyroid hormone deficiency (MMI group) in the foetus was mimicked by administrating methimazole (MMI; Sigma-Aldrich, Oakville, ON, Canada) which inhibits thyroid hormone synthesis. MMI was added to the drinking water of the pregnant dam at concentration of 0.02% (weight per volume; *w/v*), a dose used previously to induce hypothyroidism in rats (Ahmed et al., 2010; Ahmed et al., 2012). Treatment began on the first day of pregnancy (gestational day 1; GD1) and was maintained until rat pups were used for experimentation at postnatal day 14 or 15 (P14-P15). Low concentrations of salt (NaCl: 0.05%) were added to the treated water to make the MMI solution palatable; technically, this group should be termed "vehicle" but will be referred to as "controls" to avoid confusion with saline injected pups in subsequent experiments. Daily monitoring of water consumption was performed in the MMI-treated dams (~35 ml/day) and revealed no difference compared to normal water consumption in pregnant dams (~40 ml/day)(Uriu-Hare et al., 1995).

2.3. Thyroid hormone measurements

Efficiency of the MMI treatment was determined in the mothers and their pups. To minimise stress during pregnancy, maternal blood samples were taken 24 h after their pups were taken for experimentation; total thyroxine (TT4) was quantified in MMI-treated dams and controls to obtain a broad assessment of thyroid function. Sera from the offspring were taken following plethysmographic recordings; here, we measured free thyroxine (FT4) levels to compare the bioactive form of the hormone in each experimental group (MM and controls). In dams and pups, terminal blood samples (~1 ml) were collected through intracardiac puncture under deep anesthesia (ketamine/xylazine; i.p.). Blood was then transferred into a serum gel Z/1.1 microtube (Thermo Fisher Scientific, Ottawa, ON, Canada). Blood serum was separated by centrifugation at 13000 r.p.m. and 4 °C for 15 min before being stored at −80 °C. TH measurements were performed by electro-chemiluminescence (Elecsys, Modular E170, Roche Diagnostics, Mannheim, Germany) by the clinical biochemistry service of our institution (Institut Universitaire de Cardiologie et Pneumologie de Québec, Québec, QC, Canada). The measuring ranges are: T4: 5.4–320 nmol/l; FT4: 0.3–100 pmol/l.

2.4. In-vivo respiratory recordings in intact (non-sedated) pups

Respiratory activity was recorded using whole body, flow-through plethysmography as previously described for newborn rat pups (Gulemetova and Kinkead, 2011; Niane and Bairam, 2011). Briefly, the gas flow through the experimental chamber was measured with a mass flowmeter (TSI model 4140, Shoreview, MN, USA) and set at about 200 ml/min. Temperature in the chamber was maintained at 28 °C using a temperature control system (Physitemp, Clifton, NJ, USA). Calibration of the system was performed by rapidly injecting 1.5 ml of air into the chamber with a syringe. Respiratory frequency (f_R) and tidal volume (V_T) were recorded from the plethysmograph signal using a specialized data acquisition software (Labchart 8, AD Instruments, Colorado Springs, CO, USA). Barometric pressure, body temperature, chamber temperature, and humidity were measured to correct and standardize V_T and values were expressed in ml BTPS (Bartlett Jr. and Tenney, 1970; Drorbough and Fenn, 1955). These values were subsequently used to calculate minute ventilation ($\dot{V}_E = f_R \times V_T$). Composition of the gas mixtures flowing in and out of the chamber was analyzed with an oxygen analyzer (model S-3A, Ametek, Pittsburgh, PA, USA) for subsequent calculation of $\dot{V}O_2$ as an index of metabolic rate (Mortola and Dotta, 1992).

The rat pup was placed in the plethysmography chamber breathing room air (normoxia; $F_IO_2 = 0.21$). Each rat was given 10 min to acclimate and body temperature was measured by gently placing a small thermocouple for rodent inside the rectum (Harvard, Holiston, MA, USA). Once the pup appeared calm and the breathing signal became regular, breathing was then recorded for 20 min (baseline). Room air was then replaced with hypoxic gas ($F_IO_2 = 0.12$) for 20 min to record the hypoxic ventilatory response.

2.5. Laryngeal chemoreflex stimulation protocol in anesthetised pups

This procedure was based on a protocol developed previously (Baldy et al., 2017; Xia et al., 2008). Briefly, each pup was anesthetized with a mixture of urethane (1 mg/kg) and chloralose (20 mg/kg) by intraperitoneal injection. Once a surgical plane of anesthesia was reached (~20 min), the animal was placed in the supine position, a small thermistor probe was inserted into the rectum to record body temperature, which was controlled at 35 °C with a homeothermic blanket (Harvard Appratus, Holliston, MA, USA). O_2 saturation (SpO_2) and heart rate were measured continuously with pulse oxymetry (Mouse Ox, Starr Life Sciences, Oakmont, PA, USA) by placing a sensor onto the thigh of the pup. Hooked silver wires (diameter of 0.25 mm; World Precision Instrument, Sarasota, FL, USA) were introduced into the intercostal muscles in the region of the 9th–11th ribs to record electromyography (EMG) as an index of respiratory activity. A grounding wire was inserted subcutaneously into the skin over the abdomen. The EMG signal was amplified, filtered (A-M Systems, model 1800, Sequim, WA, USA), and recorded with a data acquisition system (Windaq, DataQ Instrument, Akron, OH, USA). A midline skin incision was then made in the neck and the cervical trachea was freed from adjacent tissues. The recurrent and superior laryngeal nerves were identified and carefully avoided during the dissection. The trachea was cut partially with a transverse incision so that the lumen was visible. A polyethylene tubing (PE-50, Clay Adams, Becton Dickinson Mississauga, ON, USA) was inserted slowly into the rostral part of the trachea until the tip reached the larynx. The pup's head was then tilted backward slightly to facilitate breathing *via* the opened trachea. This ensured that the water injected near the larynx drains *via* the nose and mouth and is not aspired into the lower airways and lungs.

Twenty minutes prior to recordings, animals of each groups (control and MMI) were randomly assigned to receive intraperitoneal injection of either saline (veh) or the selective $GABA_A$ receptor antagonist bicuculline (0.5 mg/kg; Tocris (Bio-Techne Canada), Oakville, ON). This dose was selected based on a previous study demonstrating the efficiency in this age group (Hiroshi et al., 2018). Once respiratory activity was stable for at least 10 min, three water injections (10 µl each) were made using a 10-µl Hamilton syringe, starting at the beginning of inspiration. A 5-min recovery period was allowed between injections. These multiple injections aimed to obtain a more representative (mean) evaluation of the reflexive responses. Apnea duration was defined as the period of apneas/respiratory disruption from the beginning of the stimulus (water injection) until the return of at least five regular, uninterrupted breaths (Xia et al., 2008). SpO_2 and heart rate were measured during baseline and throughout the laryngeal chemoreflex stimulation protocol.

2.6. Data analysis and statistics

Each group is composed of at least three litters to avoid litter specific effect. Data from male and female pups were combined since statistical analysis revealed no sex specific effect of MMI treatment and/or bicuculline injection on any of the cardiorespiratory variables considered.

Breathing variability in the non-sedated pups was calculated using the Poincaré plot method as described previously (Laouafa et al., 2017); performance analysis was determined using SD1 and SD2 (standard deviation 1–2) as variability indexes. For each animal, SD1 and SD2 were average from two periods lasting about 450 breaths on a stable portion of the baseline respiratory trace without sigh or apnea.

Anesthesia normally leads to a reduction in breathing frequency. Here, we noted that this effect was more important in MMI-treated pups than controls thus revealing a significant difference in the neural mechanisms that determine breathing frequency. To consider this aspect in our analysis of apneas, we normalised the apnea duration by first calculating the period ($T = 1/f_R$) and then dividing the apnea duration by the period (apnea/T). Based on animal studies using similar approaches (Fournier et al., 2013), the O_2 desaturation and bradycardia responses consisted of the lowest values achieved following water injection compared to baseline values. Results were considered only when pups recovered before the next injection.

Results were analyzed using a one way ANOVA for the effect of MMI treatment and bicuculline injection. ANOVA with repeated measures was used to assess the effect of time during the hypoxic ventilator response. Analysis of laryngeal chemoreflex protocol revealed that apnea duration, desaturation (SpO_2) and heart rate responses did not change with repetitive laryngeal chemoreflex stimulations. Therefore, data from the three injections were averaged and reported as a single value. None of the animals used for *in vivo* experiments were excluded from the analyses. Animals that died during the laryngeal chemoreflex protocol (2/9 MMI and 8/19 controls pups) were not considered in the analysis. Apnea duration between animals that survived the laryngeal chemoreflex stimulation and those that died before completion of the protocol were also similar for each group (alive *vs* dead; Ctrl: $P = .2115$ and MMI: $P = .6410$) and Chi-square test revealed that mortality and treatment were not linked (X^2: $P = .3051$). When ANOVA results indicated that a factor was significant ($p < .05$), a Fisher's least significant difference test was performed for *post hoc* analysis. Statistical analyses were performed using StatView 5.0 (SAS Institute, Toronto, ON, Canada). Data are reported as mean ± standard deviation (SD). ANOVA results are mainly reported in the figures legends and Table 1. Results from *post hoc* tests are represented by symbols in the table and figures.

Table 1
Comparison of body temperature and selected cardiorespiratory variables between 14 and 15 days old pups born and raised under standard conditions (Control) or born to mothers subjected to methimazole treatment (MMI; thyroid hormone deficient pups). On the day of experimentation, pups were anesthetised and injected either with saline (Veh) or with Bicuculline (Bicu; 0.5 mg/Kg). Measurements were obtained from under resting conditions. Data are reported as means ± SD. Symbols indicate results from *post hoc* tests.

	Control + Veh ($n = 13$)	MMI + Veh ($n = 6$)	Control + Bicu ($n = 13$)	MMI + Bicu ($n = 15$)	MMI effect	Bicuculline effect	Factorial interaction
Body temperature (°C)	35.6 ± 1.1	33.9 ± 0.4 *	36.5 ± 0.1†	36.5 ± 0.3†	$P < .0001$ $F_{(1,43)} = 20.89$	$P < .0001$ $F_{(1,43)} = 88.73$	$P < .0001$ $F_{(1,43)} = 20.53$
Respiratory frequency (breaths/min)	34 ± 8	12 ± 2*	40 ± 10	35 ± 9†	$P < .0001$ $F_{(1,43)} = 25.20$	$P < .0001$ $F_{(1,43)} = 28.46$	$P = .004$ $F_{(1,43)} = 9.47$
SpO_2 (%)	92 ± 4	82 ± 8*	90 ± 5	93 ± 3 *,†	$P = .02$ $F_{(1,42)} = 5.84$	$P = .006$ $F_{(1,42)} = 8.38$	$P < .0001$ $F_{(1,42)} = 23.67$
Heart rate (beats/min)	377 ± 50	194 ± 19*	396 ± 33	276 ± 33 *,†	$P < .0001$ $F_{(1,42)} = 165.83$	$P = .0001$ $F_{(1,42)} = 18.32$	$P = .01$ $F_{(1,42)} = 7.121$

* Significantly different from corresponding control value ($P = .05$).
† Significantly different from corresponding vehicle-treated value ($P = .05$).

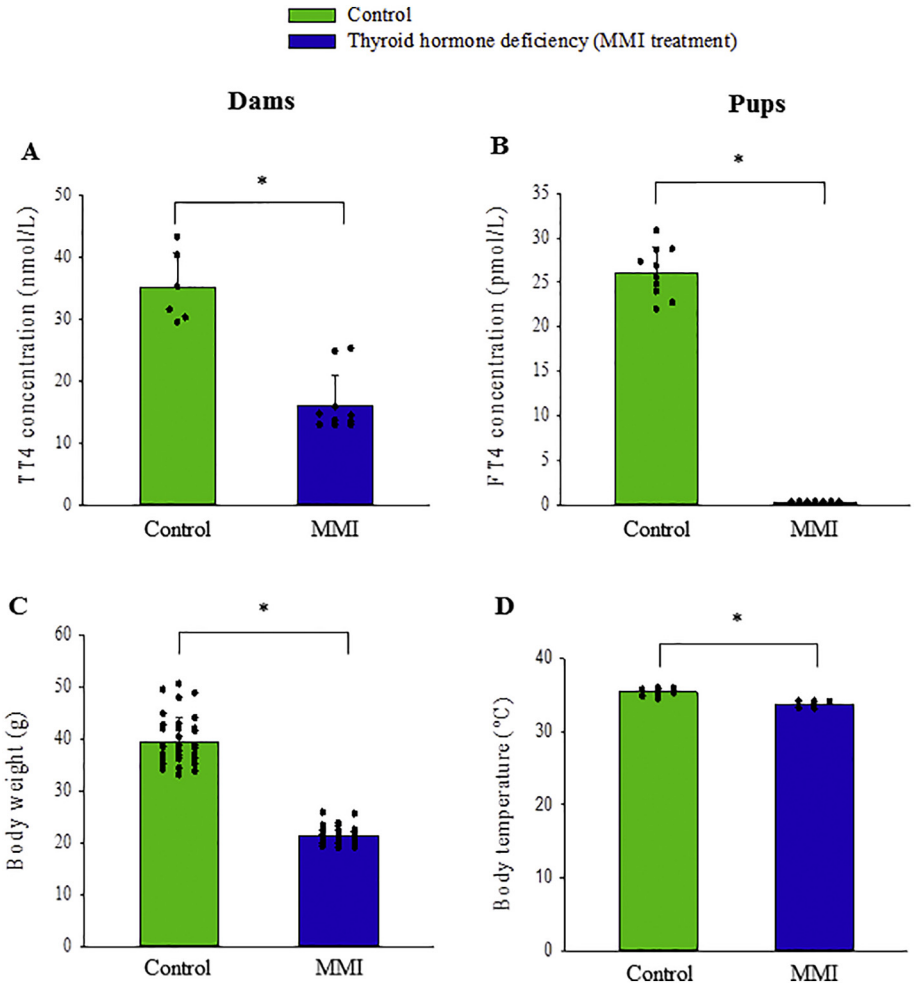

Fig. 1. Maternal methimazole treatment (MMI) reduces thyroid hormone levels and body weight. A) Comparison of total T4 blood concentration in control and MMI treated dams (MMI effect: $p = .0002$; $F_{(1,11)} = 28.770$) and B) free T4 blood concentration in control and MMI treated pups following birth (MMI effect: $p < .0001$; $F_{(1,10)} = 153.072$). C) Comparison of body weight (MMI effect: $p < .0001$; $F_{(1,43)} = 287.752$) and D) body temperature (MMI effect: $p < .0001$; $F_{(1,14)} = 44.9$) between control and TH-deficient pups (MMI treated). Data are reported as means ± SD. Symbols indicate results from *post hoc* tests. * significantly different from control group ($p = .05$).

3. Results

3.1. Efficacy of maternal methimazole treatment (MMI) on thyroxin levels (T4) and pups

Total T4 (TT4) levels measured in the dams (*post partum* day 4) revealed a -52% decrease in the sera of MMI treated mother compared to controls (Fig. 1A). MMI can pass through the placental barrier and maternal milk, and therefore also affects THs synthesis in the foetus and pups. Free T4 (FT4) levels were measured in the 14–15 days old offspring; following MMI treatment FT4 levels were below detection level of the test in all the samples (Fig. 1B). Consistent with other studies using this model (Özgür et al., 2016), thyroid hormone deficient pups weighed, on average, 50% less than controls (Fig. 1C).

3.2. Thyroid hormone deficiency attenuates the breathing frequency response to hypoxia in intact pups

The original recordings presented in Fig. 2 illustrate respiratory activity at rest (normoxia; boxed area) and the first minutes that follow the onset of hypoxic stimulation (arrow). Under resting conditions, measurements of $\dot{V}_E$, f_R, and V_T did not differ between thyroid hormone deficient pups to controls (Fig. 2A, B, and C, respectively). Thyroid hormones are known for their influence on metabolism (Ahmed et al., 2008); while body temperature of MMI-treated pups was 1.7 °C colder than controls (Fig. 1D) (33.6 °C ± 0.5 and 35.4 °C ± 0.5, respectively; $P < .0001$), neither $\dot{V}_{O_2}$ nor the convective requirement ($\dot{V}_E/\dot{V}_{O_2}$) were affect by treatment (Fig. 2D-E). Breath-by-breath analysis was performed under normoxic conditions and stability of the breathing pattern is portrayed in Poincaré plot (Fig. 2F). Evaluation of respiratory activity at rest revealed increased variability indexes (SD1 and SD2) in MMI treated pups compared to controls (Fig. 2G).

When comparing the time course of the f_R response to hypoxia (Fig. 3A) we first noticed that at the onset of the hypoxic challenge (first 6 min), the hyperpneic responses were similar between groups. Unlike controls, MMI-treated pups were then unable to sustain the initial f_R increase over the entire hypoxic period. As a result, the response measured during the "steady state" phase (last 5 min) was 69% lower in MMI-treated pups than controls (Fig. 3B). The V_T and $\dot{V}_E$ responses were unaffected by treatment (Fig. 3C, D). While the anapyrexic response of MMI pups was slightly larger than controls (−2.0 °C ± 1.1 *versus* − 1.1 °C ± 0.6, respectively; $P = .06$), the drop in $\dot{V}_{O_2}$ did not differ between groups (−53% ± 15 *versus* − 60% ± 11 for control and MMI, respectively; $P = .4$). Consequently, the hypoxic $\dot{V}_E/\dot{V}_{O_2}$ values were similar between groups (63 ± 18 *versus* 66 ± 27 for control and MMI, respectively; $P = .8$).

3.3. Effect of anesthesia and thyroid hormone deficiency on cardiorespiratory activity at rest and in responses to laryngeal chemoreflex stimulation

While respiratory activity of MMI and control pups did not differ under "standard" (non-sedated) conditions, anesthesia revealed significant effects of treatment. Comparison of the original recordings reported in Fig. 4A and C illustrate the striking differences in basal cardiorespiratory activity between MMI and control pups. Prior to laryngeal chemoreflex stimulation, body temperature and all cardiorespiratory variables of MMI-treated pups were significantly lower than

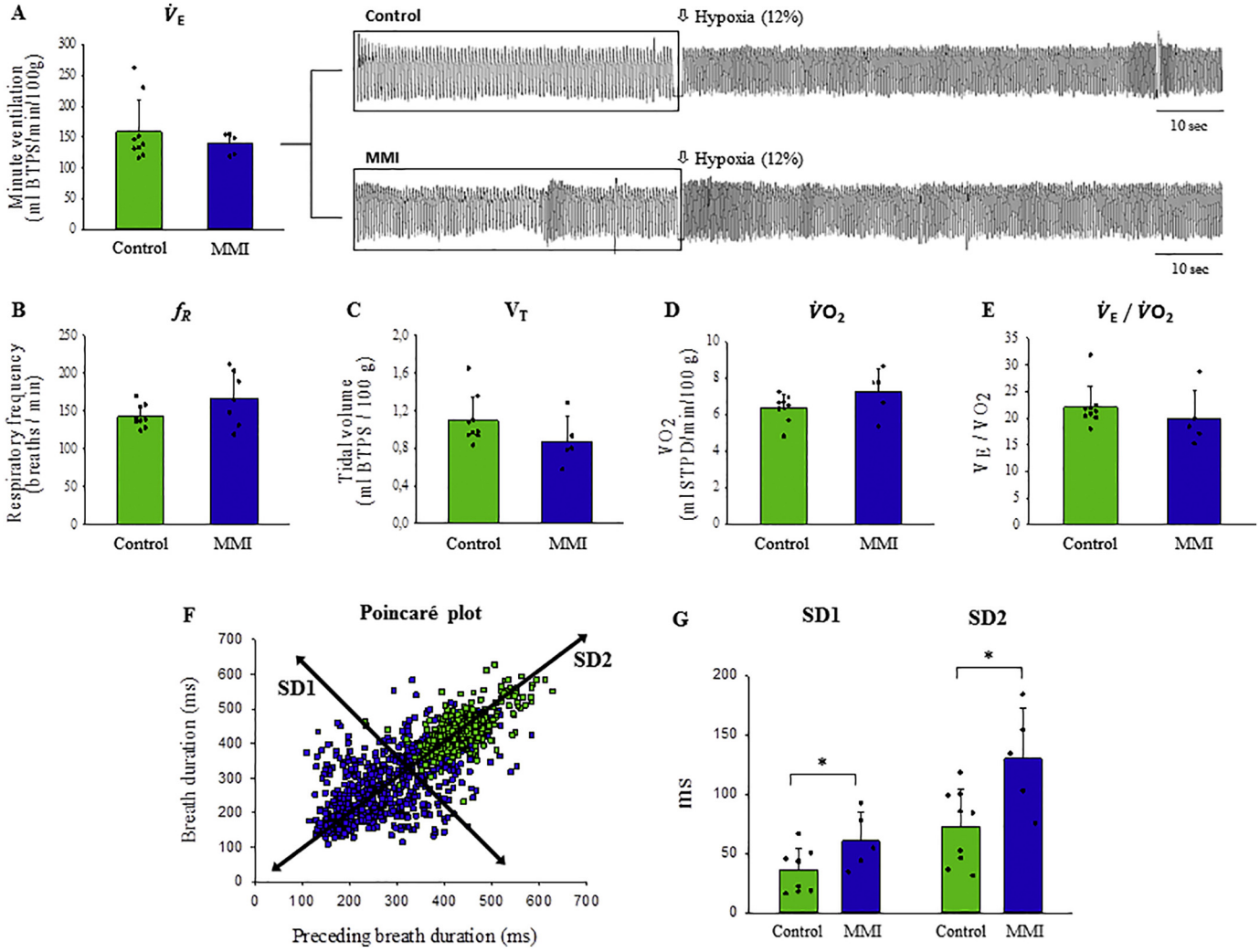

Fig. 2. Whole body plethysmography in unrestrained rats. Thyroid hormone deficiency does not affect basal respiratory and metabolic variables measured in awake pups (P14–15). Between group comparisons of A) minute ventilation ($\dot{V}_E$) (MMI effect: $p = .7158$; $F_{(1,12)} = 0.140$), B) respiratory frequency (f_R) (MMI effect: $p = .0975$; $F_{(1,12)} = 3.279$), C) tidal volume (V_T) conditions (MMI effect: $p = .2254$; $F_{(1,12)} = 1.650$), D) O_2 consumption ($\dot{V}O_2$) (MMI effect: $p = .1845$; $F_{(1,12)} = 2.004$), and E) convective requirement ratio ($\dot{V}_E/\dot{V}O_2$) (MMI effect: $p = .4037$; $F_{(1,12)} = 0.754$). F) Typical example of a Poincaré plot showing individual breath duration as a function of the duration of the preceding breath (both in milliseconds). Arrows indicate the line on which standard deviations (SD) are calculated. G) Between group comparisons of SD1: perpendicular to identity (MMI effect: $p = .0473$; $F_{(1,12)} = 4.881$) and SD2: identity (MMI effect: $p = .0134$; $F_{(1,12)} = 8.387$). Data are reported as means ± SD. Symbols indicate results from *post hoc* tests. * significantly different from control group ($p = .05$).

controls (Tb: −1.7 °C; f_R: −60%; SpO_2: −11%; Heart rate: −49%) (Table 1; Fig. 4A and C). In control pups, inactivation of $GABA_A$ receptors by bicuculline had very limited effects on basal physiological variables; only body temperature was increased (+0.9 °C). The recording also show that the effects of bicuculline injection were more important in MMI-treated pups (Fig. 4); this treatment increased body temperature and cardiorespiratory variables to values similar to those observed in control pups (body temperature: +2.6 °C; f_R: +61%; SpO_2: +11%; Heart rate: +31%) (Table 1; Fig. 4). The significant factorial interactions (MMI x bicuculline) support this observation (Table 1).

The recordings presented in Fig. 4 also show that water injection near the larynx activated the laryngeal chemoreflex and provoked significant cardiorespiratory inhibition in both groups. However, the apneas measured in MMI treated pups were 333% longer than controls (Fig. 5A). Bicuculline injection in control groups did not affect this response, but in MMI pups, this treatment shortened apnea duration by −38% (Fig. 5A). Basal respiratory activity influences apnea duration. To consider the between-group differences in basal f_R in this evaluation, we then expressed apnea duration as a function of the f_R measured before laryngeal chemoreflex stimulation (apnea duration/period). This normalization brought the apneic response of MMI-treated pups to a level similar to controls; yet, the apnea duration/period ratio of MMI pups injected with bicuculline remained greater than vehicle-injected pups (Fig. 5B). The desaturation response (SpO_2 drop) was 82% greater in thyroid hormone deficient pups than controls; however, bicuculline treatment had not effect on this response (Fig. 5C). The laryngeal chemoreflex-induced bradycardia was not affected by either MMI treatment or bicuculline injection (Fig. 5D).

4. Discussion

Thyroid hormone levels of infants born to mother with hypothyroidism (especially those born preterm) are significantly low at birth, and it is now established that this population is more likely to require respiratory care during early life (Biswas et al., 2002; Casey et al., 2005; Männistö et al., 2013). Thyroid hormones regulate several processes but owing to their primary role in brain development, we hypothesized that deficiency in thyroid hormones disrupts the neural network that regulates cardiorespiratory function and thus predispose rat pups to disease. Under resting (non-sedated) conditions, respiratory function of

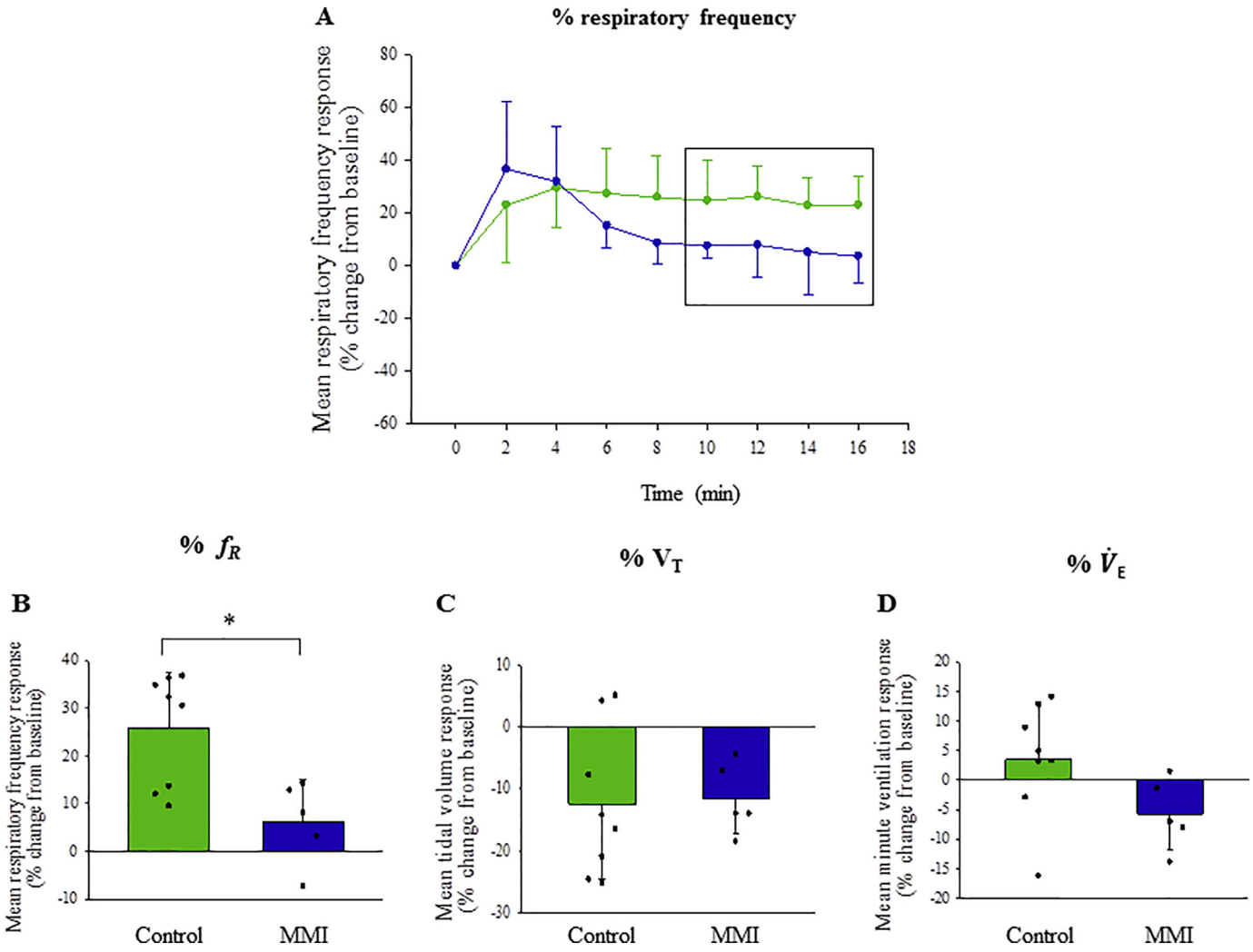

Fig. 3. Ventilatory response to a 12% hypoxic event between control and MMI-treated pups. A) Respiratory frequency response over the 15 min of hypoxia (MMI effect: $p = .6102$; $F_{(1,9)} = 0.285$) (Time effect = $p < .0001$; $F_{(1,14)} = 6.097$) (Factorial interaction: $p < .0001$; $F_{(1,14)} = 10.361$). Box area in panel A represents the last 5 min of hypoxic exposure where physiological responses were determined for B) Respiratory frequency (f_R) (MMI effect: $p = .0089$; $F_{(1,9)} = 10.040$), C) tidal volume (V_T) (MMI effect: $p = .5499$; $F_{(1,9)} = 0.386$) and D) minute ventilation ($\dot{V}_E$) (MMI effect: $p = .1181$; $F_{(1,9)} = 2.985$) responses in the last 5 min of hypoxic exposure. Data are reported as means ± SD. Symbols indicate results from *post hoc* tests. * significantly different from control group (p = .05).

thyroid hormone deficient pups appeared normal as none of the respiratory parameters differed from controls even though body temperature was lower, as expected. This suggests a potential specificity of thyroid hormone deficiency treatment on GABAergic activity in cardiorespiratory circuits. However, breath-to-breath variability during normoxia was higher in MMI treated pups. Variability in ventilation can originate from intrinsic properties of the respiratory rhythm generator and/or sensory afferents influencing its action, including cardiac output, cerebral blood flow, chemoreflex gain, and chemical drive (Hilaire and Duron, 1999; Khoo, 2000). Moreover, exposing pups to a respiratory challenge or anesthesia revealed important deleterious consequences of thyroid hormone deficiency on the cardiorespiratory control system. Together, these results support our hypothesis and highlight the cardiorespiratory vulnerabilities associated with perinatal thyroid hormone deficiency. These observations are significant as they indicate that thyroid hormones are important players in the pathophysiology of cardiorespiratory disorders of the newborn related to neural control dysfunction, including apnea of prematurity (AoP) and sudden infant deaths syndrome (SIDS). Results from pharmacological experimental indicate that potentiation of GABAergic modulation is an important pathophysiological mechanism.

4.1. Critique of the model

Evaluating the pathophysiological/clinical relevance of any experimental model must be done cautiously owing to the difficulty of transposing human standards to animals. Pharmacological treatment of gestating dams reduced total T4 levels by ~50%. This, in turn, virtually eliminated circulating free T4 in pups. In humans, hypothyroidism during gestation is considered clinically significant when values are below the 5th percentile which, depending on the stage of the pregnancy, represents a reduction well below the one reported here (Alexander et al., 2017; Elmlinger Martin et al., 2001). Gestational hypothyroidism is a significant risk factor for preterm birth, which in itself can impose an additional thyroid hormone deprivation to the offspring. Our total T4 measurements and the fact that none of our MMI treated dams gave birth prematurely indicate that the thyroid hormone deficiency experienced by gestating dams is relatively modest. We know, however, that this protocol is sufficient to reduce birth weight and interfere with respiratory rhythm generation in newborn (Rousseau and Kinkead, 2016).

Infants and rats born at term show a significant postnatal elevation of thyroid hormones that then decline progressively over the following days (Abuid et al., 1973; Biswas et al., 2002; Dussault and Labrie, 1975;

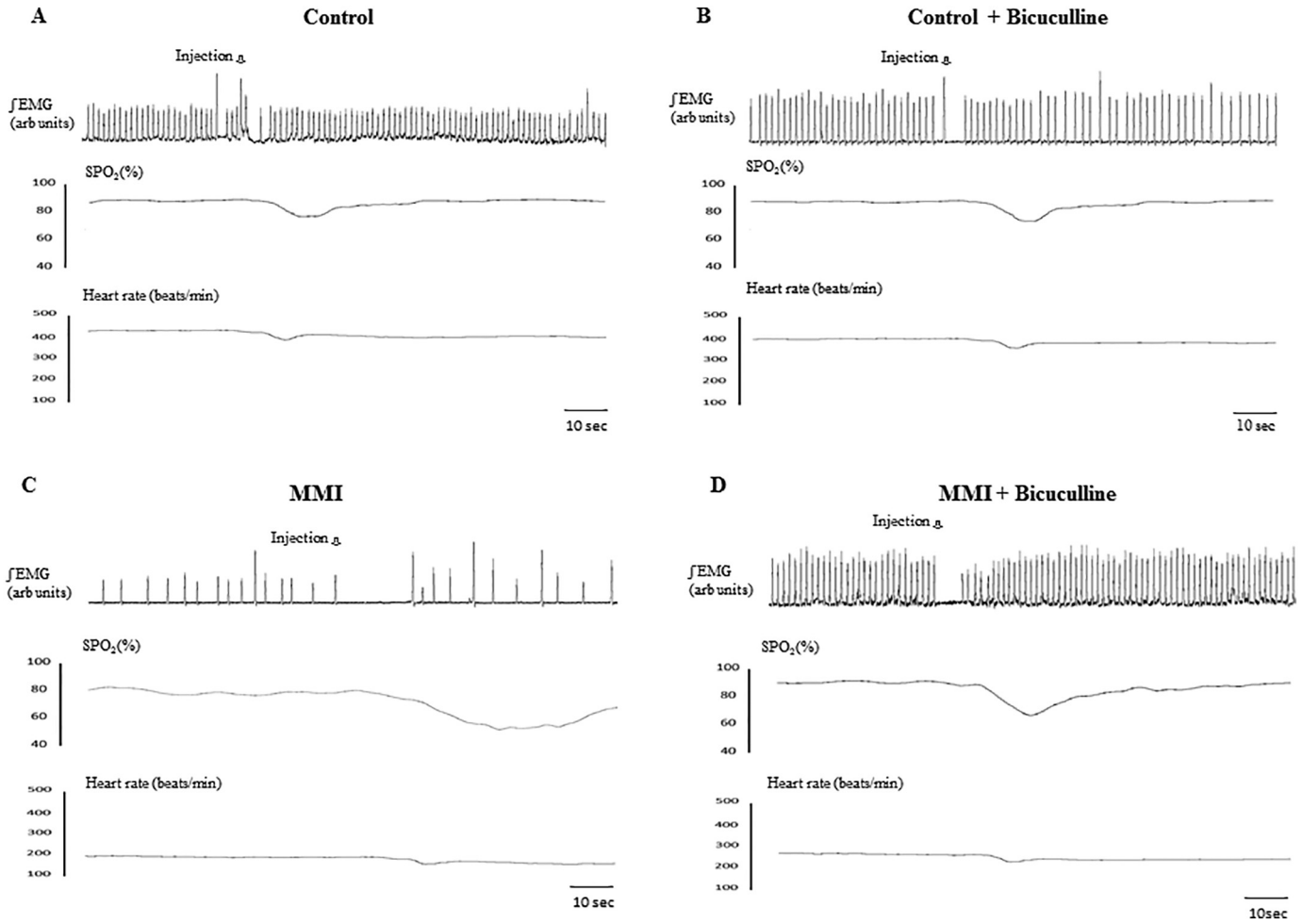

Fig. 4. Original recording comparing cardiorespiratory responses to stimulation of the laryngeal chemoreflex by water injection (10 μl) near the larynx between pups subjected to A) control conditions, B) control conditions + bicuculline injection before recording, C) TH deficiency in the form of MMI treatment and D) TH deficiency (MMI) + bicuculline injection before recording. In each panel, the traces illustrate (from top to bottom): intercostal EMG, SpO_2, and heart rate.

LaFranchi, 1999). Preterm birth interferes with this process (LaFranchi, 1999) and maintaining MMI treatment following birth aimed to reproduce this aspect of thyroid hormone deficiency. Considering that the free T4 level of thyroid hormone deficient pups were below the detection threshold of the test used and that the cut off for treatment in the clinic is 7.7 pmol/L and the normal range is 11–30 pmol/L (~38% drop) the effect of the treatment used here may be severe (LaFranchi, 2011). In light of the effects of thyroid hormones on lung development, surfactant synthesis and diaphragm muscle (Ballard, 1989; Holt et al., 1993; Ianuzzo et al., 1984; Massaro et al., 1986), this then raises the possibility that our protocol had broad (non specific) effects on the respiratory system rather than its control network. Measurements of arterial blood gas would ensure that the MMI treatment does not compromise gas exchange but in the absence of such results, quantification of metabolic demand and baseline breathing are reliable indicators and the lack of treatment effect indicates that if MMI-treatment had non-specific effects, the physiological impact is limited. Furthermore, the tidal volume data obtain in both groups do not support a reduction in diaphragm function following thyroid hormone deficiency. While the reduced body temperature in MMI-treated pups could modulate laryngeal response to stimulation under anesthesia (Curran et al., 2005; Xia et al., 2008), comparison between anesthetised and intact pups show that the impact of temperature on resting breathing frequency was not proportional between preparations, thus pointing to an alternate mechanism than body temperature. Together, these observations and the fact that anesthesia and stimulation of respiratory reflexes was necessary to reveal the effects of thyroid hormone deficiency argue that disruption of the neural control network is the main reason why respiratory anomalies were observed in thyroid hormone deficient pups.

4.2. Hypoxic ventilatory response is attenuated thyroid hormone deficient pups

Analysing the time course of the hypoxic ventilatory response provides insight about the relative contribution of the different components of the respiratory control system (Powell et al., 1998). The rapid rise in respiratory frequency at the onset of hypoxia reflects activation of peripheral O_2 chemoreceptors and our results do not suggest that MMI-treatment affected carotid body function in pups. The subsequent changes that take place when hypoxia is maintained reflect neurological and metabolic adaptions. The hypoxic ventilatory response undergoes significant changes during early life (Mortola, 2001). When facing sustained hypoxia, immature/newborn mammals will generally conform to the reduced O_2 availability by lowering $\dot{V}o_2$ and ventilation. This response contrasts with the response of adults that minimise metabolic changes and aim to maintain O_2 supply by a sustained hyperventilation. Based on the temperature and $\dot{V}o_2$ responses, thyroid hormone deficiency did not affect metabolic response to hypoxia. The inability to sustain a hyperpnea through the stimulation period is suggestive of an abnormal central response potentially driven by diverse changes in neurochemical levels including GABA and adenosine, both

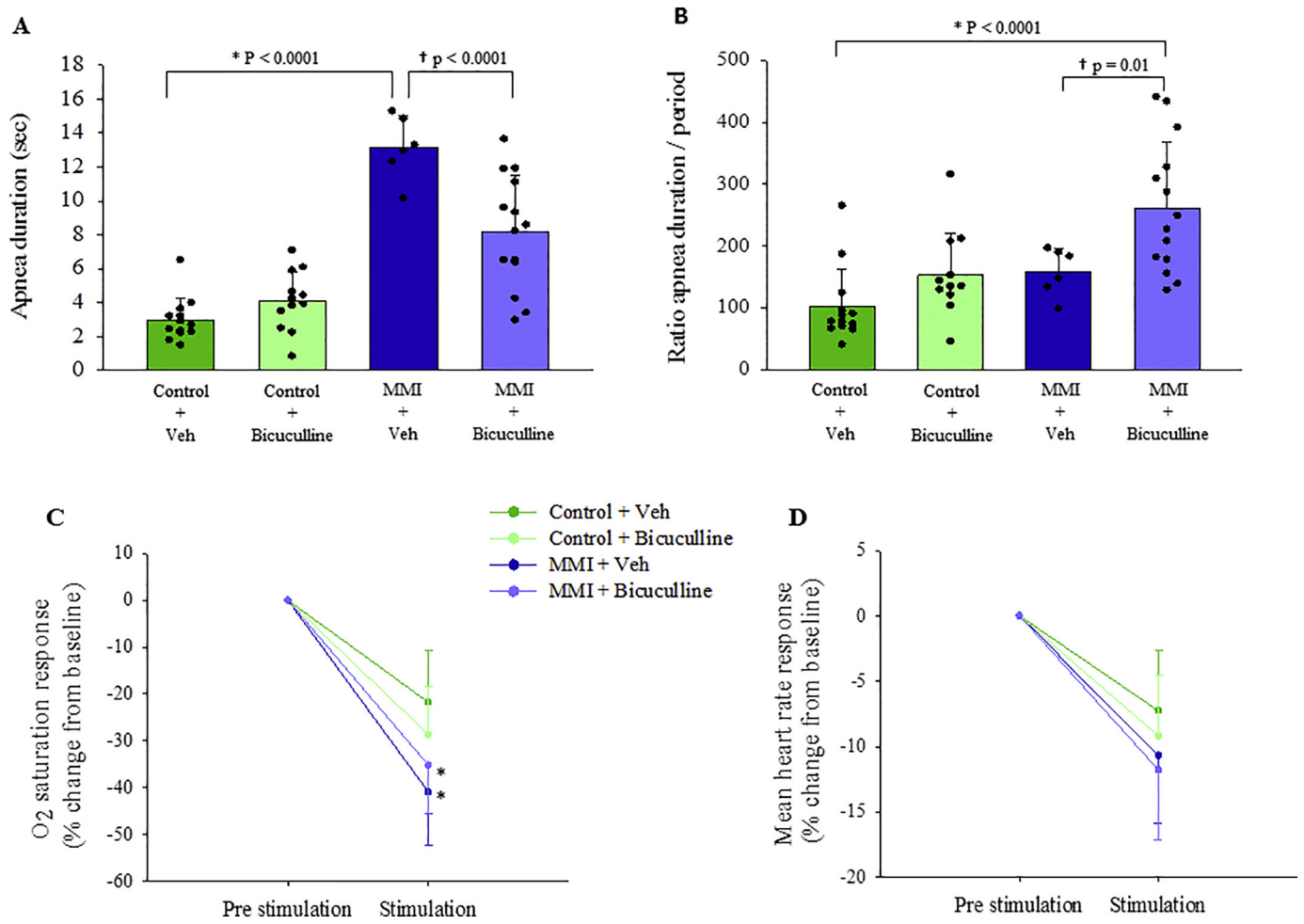

Fig. 5. Thyroid hormone deficiency augments cardiorespiratory inhibition induced by laryngeal chemoreflex stimulation. A) Combined apnea duration (MMI effect: $p < .0001$; $F_{(1,40)} = 96.642$) (Bicuculline effect: $p = .0024$; $F_{(1,40)} = 10.425$) (Factorial interaction: $p < .0001$; $F_{(1,40)} = 21.623$), B) apnea duration normalised to the duration of the breathing cycle (ratio apnea duration/period) (MMI effect: $p = .0004$; $F_{(1,40)} = 14.921$) (Bicuculline effect: $p = .0386$; $F_{(1,40)} = 4.558$) (Factorial interaction: $p = .8357$; $F_{(1,40)} = 0.044$), C) combined SpO_2 (MMI effect: $p = 0{,}0006$; $F_{(1,40)} = 13.831$) (Bicuculline effect: $p = .8666$; $F_{(1,40)} = 0.029$) (Factorial interaction: $p = .0756$; $F_{(1,40)} = 3.328$) and D) combined heart rate (MMI effect: $p = .0544$; $F_{(1,40)} = 3.927$) (Bicuculline effect: $p = .3228$; $F_{(1,40)} = 1.002$) (Factorial interaction: $p = .7946$; $F_{(1,40)} = 0.069$) responses observed in control, control + bicuculline, MMI and MMI + bicuculline groups. Data are reported as means ± SD. Symbols indicate results from *post hoc* tests. * significantly different from control group (p = .05). †significantly different from corresponding vehicle-treated value (p = .05).

modified by thyroid hormone availability (Ahmed et al., 2008). Thus, the lower frequency response observed in thyroid hormone deficient pups indicates that the neural control network of these animals is less mature than controls, an effect consistent with the role of thyroid hormones on brain development.

4.3. Thyroid hormone deficiency augments anesthesia-induced respiratory depression

In the anesthetized rat, body temperature, respiratory frequency, oxygen saturation, and heart rate were all reduced in normal conditions by perinatal MMI treatment. Except for body temperature, these observations are quite different from similarity of respiratory variables in control and MMI-treated pups under intact conditions. Such striking effect of anesthesia was unexpected; however, it shows that cardiorespiratory function of thyroid hormone deficient rat pups is very vulnerable once the respiratory drive related to wakefulness is eliminated. Urethane and chloralose both potentiate GABAergic currents (Garrett and Gan, 1998; Hara and Harris, 2002). Here, we showed that inactivation of $GABA_A$ receptors by bicuculline restores body temperature and cardiorespiratory values of anesthetised thyroid hormone deficient pups to near normal level but has limited effects in controls. These data indicate that thyroid hormone deficiency potentiated this inhibitory neurotransmission and that despite being injected intraperitoneally, bicuculline can act on brain regions regulating cardiorespiratory functions. $GABA_A$ receptors being ubiquitous in the hypothalamus, brainstem, and medulla, the present data does not provide the information allowing us to propose a specific site of action. Changes in temperature alone can influence breathing and heart rate but a 1.7 °C difference is not sufficient to account for the large changes reported here (Schaeffer, 1998). We therefore propose that the treatment-related effects are mainly due to an enhanced GABAergic inhibition in regions regulating cardiorespiratory function. This effect could be direct or *via* actions on structures regulating arousal states, including the serotonergic and/or the orexinergic system. This interpretation is based on the consequence of thyroid hormone deficiency on the development of the GABAergic system in the cerebral cortex and the hippocampus (Friauf et al., 2008; Gilbert et al., 2007). Deletion of thyroid hormone receptors ($Tr^{-/-}$) in mice is also associated with a decrease in body temperature and heart rate, a result in agreement with our data from perinatal thyroid hormone deficiency in rats (Forrest and Vennström, 2000). Interestingly, baseline SpO_2 levels in thyroid hormone deficient rats (82% O_2) were close to the 80% limit considered as clinically significant for adverse effects in infants (Eichenwald et al., 2016). With such low SpO_2, MMI-

treated pup are potentially hypoxemic. The reduced response to hypoxia discussed previously may contribute to the abnormally low breathing frequency that characterized this group (Fig. 3A).

4.4. The GABAergic regulation of the laryngeal chemoreflex following thyroid hormone deficiency

An abnormal or immature laryngeal chemoreflex can lead to prolonged, life threatening apneas and severe bradycardias. It is not surprising therefore that an excessive laryngeal chemoreflex has been associated with sudden infant death (Kinney and Thach, 2009). Such profound cardiorespiratory depression generally reflects the activation of powerful inhibitory signals converging onto the cardiorespiratory network during early life (Praud, 1999; Xia et al., 2013). This principle was observed in our experiments where laryngeal chemoreflex-induced apneas were longer in MMI treated pups than controls. This strong response was concomitant to more profound O_2 desaturation. Since apnea duration is greatly determined by basal respiratory frequency, we expressed these results as function of basal breathing frequency. With this approach, apnea duration was now similar between groups but interestingly, this normalization procedure showed that bicuculline treatment prolonged laryngeal chemoreflex-induced apneas in thyroid hormone deficient pups but not controls. The lack of effect in controls is consistent with other observations in this group prior to laryngeal chemoreflex stimulation. However, the prolonged apneas in thyroid hormone deficient pups treated with bicuculline are difficult to reconcile with the overall increase in body temperature and cardiorespiratory function observed prior to laryngeal chemoreflex stimulation. They suggest, however, that the influence that GABAergic neurotransmission exerts on laryngeal chemoreflex responses is different and could take place more selectively by acting on distinct structure that regulates the apneic response. Because it receives numerous sensory afferents from the laryngeal mucosa, the caudal region of the NTS could be contributing to this differential effect. Conversely, neither the O_2 desaturation nor the bradycardia responses triggered by laryngeal chemoreflex stimulation were affected by bicuculline treatment in MMI treated pups. Based on the rationale developed previously, this indicates that other structures are involved; the nucleus ambiguous and motor nucleus of the vagus are potential candidates owing to their role in the cholinergic regulation of cardiovascular function.

5. Conclusion

Adequate supply of thyroid hormones is essential to brain development during early life. Owing to its multiple roles, thyroid hormone deficiency can compromise several aspects of maternal and infant health; however, the results reported here provide support to the hypothesis proposing that this condition can contribute to respiratory disorder by predominantly compromising the function of the cardiorespiratory control network. Thyroid hormone supplementation is frequently used in the clinic, but these data provide the basis necessary to evaluate the therapeutic values of this treatment to address respiratory disorders related to neural control dysfunction more specifically.

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Declaration of interests

None.

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