Published in final edited form as:

Respir Physiol Neurobiol. 2014 March 1; 193: 29–37. doi:10.1016/j.resp.2014.01.005.

Hypothyroidism Affects D2 Receptor-mediated Breathing without altering D2 Receptor Expression

Evelyn H. Schlenker^{1,*}, Rodrigo Del Rio², and Harold D. Schultz²

¹Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, 414 East Clark St., Vermillion, SD 57069

²Department of Cellular & Integrative Physiology, University of Nebraska College of Medicine, 985850 Nebraska Medical Center, Omaha, NE 68198-5850

Abstract

Bromocriptine depressed ventilation in air and D2 receptor expression in the nucleus tractus solitaries (NTS) in male hypothyroid hamsters. Here we postulated that in age- matched hypothyroid female hamsters, the pattern of D2 receptor modulation of breathing and D2 receptor expression would differ from those reported in hypothyroid males. In females hypothyroidism did not affect D2 receptor protein levels in the NTS, carotid bodies or striatum. Bromocriptine, but not carboxirole (a peripheral D2 receptor agonist), increased oxygen consumption and body temperature in awake air-exposed hypothyroid female hamsters and stimulated their ventilation before and following exposure to hypoxia. Carboxirole depressed frequency of breathing in euthyroid hamsters prior to, during and following hypoxia exposures and stimulated it in the hypothyroid hamsters following hypoxia. Although hypothyroidism did not affect expression of D2 receptors, it influenced central D2 modulation of breathing in a disparate manner relative to euthyroid hamsters.

Keywords

Dopamine D2 receptors; female; hypothyroidism; control of breathing; hypoxia; bromocriptine; carboxirole

1.0 Introduction

Hypothyroidism is a common endocrine disorder. Moreover, hypothyroidism is more prevalent among women and increases with age. For example, Empson and coworkers noted that of 3504 subjects over the age of 49 years, hypothyroidism was present in 7.1% of women and 3.7% of men (Empson et al., 2007). Lucas and coworkers reported a prevalence of hypothyroidism of 8.9% with 71% of hypothyroid individuals being women (Lucas et al., 2010). In a large population study in Colorado, Carnais and coworkers (Canaris et al., 2000) reported a higher prevalence of women with hypothyroidism than men that increased with

*Corresponding Author. Phone: 605-677-6150, FAX: 605-677-6381, Evelyn.Schlenker@usd.edu.

EHS conducted the ventilatory and metabolic studies and wrote the paper, RDR conducted and evaluated D2 Western Blot expression in the carotid bodies and brain regions, and HDS helped write the paper.

age. Thus, females are more likely to develop hypothyroidism than males which becomes more common with age.

Clinically, hypothyroidism causes depression of breathing and contributes to the development of sleep apnea, insulin insensitivity and increases the risk for cardiovascular disease (Braverman and Utiger, 2005) (Kansagra et al., 2010; Lakshmi et al., 2009) (Mainenti et al., 2010). Moreover, the development of sleep apnea includes an interplay of many players including factors that globally or selectively (i.e. diaphragm versus upper airway muscles) diminish central nervous system drive, affect the function of reflexes involving inputs from mechanoreceptors as well as chemoreceptors (such as the carotid body) as well as changes in various neurotransmitter and receptor levels. These include gamma aminobutyric acid, acetylcholine, serotonin, norepinephrine and dopamine (see recent review by Ramirez, J-M, et al, (Chenuel et al., 2005; Dempsey et al., 2012; Ramirez et al., 2013).

Dopaminergic receptors are G protein coupled receptors can be divided into two groups D1-like that result in stimulatory responses and D2-like receptors (D2 and D3) whose stimulation cause depression (Beaulieu and Gainetdinov, 2011). Dopaminergic D2 receptors are located in the carotid bodies and also several brain regions associated with control of breathing, including nucleus tractus solitaires (NTS), the paraventricular nucleus of the hypothalamus (PVN) and in the striatum ((Bairam and Carroll, 2005; Hyde et al., 1996; Nobrega et al., 1996) Both the NTS and the PVN are integratory regions involved with modulating hypoxic responses and autonomic function (Reddy et al., 2005). Although less investigated, several studies have shown that the striatum is involved in regulation of breathing (Evans et al., 1999; Nattie et al., 2001).

Stimulation of D2 receptors generally depress ventilation. For example, Nielsen and Bisgard (Nielsen and Bisgard, 1984) administered bromocriptine, a D2 receptor agonist, intravenously to decerebrate, vagotomized, paralyzed, carotid body denervated air breathing dogs. Following administration of the drug, the investigators reported decreased phrenic nerve amplitudes without an effect on the frequency of bursts. Co-administration with the D2 receptor antagonist haloperidol with bromocriptine prevented these effects. More recently Lalley and coworkers (Lalley and Mifflin, 2012) investigated the effects of piribedil, a D2/D3 receptor agonist on phrenic nerve response in anesthetized, paralyzed and ventilated cats whose vagi were cut. Results indicated that in dose dependent manner intravenously administered piribedil depressed peak action potential frequency and rate of discharge. Most studies suggest that D2 receptors depress breathing, although a few actually had an excitatory effect of D2 receptors on ventilation (Burton and Kazemi, 2000). Moreover, the D2 receptors can affect ventilation by modulating the carotid body activity (Kumar and Prabhakar, 2011; O'Halloran et al., 1998).

Hypothyroidism also has profound effects on the expression and function of various neurotransmitters that can modulate breathing. For example, Schlenker and coworkers showed that naloxone (a mu opioid receptor antagonist) caused depression of breathing in male hypothyroid hamsters but stimulated breathing in euthyroid males (Schlenker et al., 1994). Varney and Schlenker (Varney and Schlenker, 2007) reported that a specific

serotonin 2A receptor antagonist resulted in long-term depression of ventilation following intermittent hypoxia relative to baseline values in male euthyroid, but not in hypothyroid hamsters. More recently, studies from our laboratory demonstrated that hypothyroidism induced for 5 months in male hamsters had profound effects on D2 receptor modulation of breathing especially following exposure to hypoxia (Sykora et al., 2013). Specifically administration of the D2 receptor agonist bromocriptine (relative to vehicle-treatment) depressed ventilation in both groups exposed to air or to hypoxia, but hypothyroid bromocriptine –treated hamsters increased ventilatory responsiveness to hypoxia, while euthyroid hamsters decreased ventilatory responsiveness to hypoxia and exhibited a post-hypoxic depression. Moreover, in that study, hypothyroidism increased D2 receptor levels in the nucleus tractus solitaries (NTS), but D2 receptor levels were comparable in the striatum and carotid bodies of both groups of hamsters. Interestingly, the ventilatory and D2 receptor results were dissimilar to those previously reported in younger male hypothyroid hamsters (Schlenker and Schultz 2011). In that study bromocriptine had no effect on ventilation during air exposure in hypothyroid or euthyroid hamsters, but depressed ventilation following hypoxic exposures in the euthyroid animals while stimulation it in the hypothyroid hamsters. Moreover, hypothyroid males exhibited increased D2 receptor protein levels in the striatum and CB's, but decreased levels in the paraventricular hypothalamic nucleus relative to euthyroid hamsters. No effect of hypothyroidism was seen on D2 receptor levels in the NTS. Thus, age may also affect hypothyroidism dopaminergic mediated control of breathing and dopamine receptor expression.

What effects hypothyroidism has on D2 receptor expression and D2 receptor modulation of breathing in female hamsters is the purpose of the present study. We postulated that the pattern of ventilatory responses to a central D2 receptor agonist bromocriptine would be different to that previously found in the older hypothyroid males. In the present study we also used carboxirole, a peripherally acting D2 receptor agonist to determine if carotid body D2 receptor regulation of breathing was altered by hypothyroidism in female hamsters. Moreover, we posited that in hypothyroid females the expression of D2 receptors in the striatum, NTS, and the carotid bodies should be increased relative D2 expression in the same regions of euthyroid hamsters.

2.0 Methods

2.1 Animals

Seven to eight week old female golden Syrian hamsters were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Ten animals received propylthiouracil (PTU) in drinking water to induce hypothyroidism as described below and 9 received tap water. This is a technique previously used in our laboratory (Schlenker and Schultz, 2011) and resulted in decreased serum thyroid hormones levels. In both experiments animals were housed three to four per cage, exposed to 14 hours of light and 10 hours of dark, and received rodent pellets (8604, Harlan Sprague Dawley, Inc.). Food and fluids (tap water or PTU in tap water) were available ad libitum. Five months following the start of PTU administration the experiments outlined below were started. Three days prior to commencement of the experiments, hamsters were acclimated to the barometric chamber for 30 minutes each day. The

University of South Dakota Animal Care and Use Committee in accordance to the “Guide for the Care and Use of Laboratory Animals” approved all procedures used in these studies.

2.2 Ventilatory and Metabolic Measurements

Oxygen consumption and respiratory measurements were made in hamsters placed into a 20.2×7.9 cm Plexiglas cylindrical chamber. Ventilatory measurements used the barometric method previously reported in hamsters (Schlenker, 1984). Ports allowed air or 10% oxygen in nitrogen (hypoxia) to enter and exit the chamber. Chamber pressure was measured using a Statham pressure transducer and airflow rate through the chamber was measured using a Gilmont rotameter. The barometric pressure was measured using a W.M. Welch (Chicago, IL) mercury barometer. Chamber temperature was measured using a Fisher Scientific digital thermometer and the relative humidity within the chamber averaged 50%. The average of 15–20 Inspiratory and expiratory times were measured and were averaged for each portion of the study. Frequency of breathing was calculated by adding inspiratory and expiratory times and dividing the sum into 60 (sec/min). The minute ventilation was calculated by multiplying frequency of breathing by tidal volume. Tidal volume and minute ventilation were normalized by body weight (variable*1000/BW). Oxygen consumption was determined by subtracting the fractional content of O₂ in the output air from the fractional content of O₂ in the input air and multiplying the difference by the flow rate. Values were corrected to STPD and by body weight as described for the ventilatory parameters.

2.3 Protocols for Ventilatory Studies

For the carboxirole study the hamster was weighed and injected subcutaneously with either vehicle (5% DMSO in saline) or 0.2 mg/kg carboxirole in vehicle (Tocris Bioscience, Ellisville, MO). The dose of carboxirole was obtained from previous studies in our laboratory (Schlenker, 2007). Each hamster received vehicle or carboxirole in a random manner at the same time of day with 2 days between treatments. Following drug administration, the hamster was placed into the chamber for 35 minutes and exposed to room air. During the last minute of air exposure oxygen consumption, frequency, tidal volume, and minute ventilation were determined. The hamster was then exposed to hypoxia for 5 minutes. During the last minute of exposure to hypoxia, tidal volume, frequency and minute ventilation were determined. Following the hypoxic exposure, the chamber was washed out with room air for 5 minutes. During the last minute of the washout, tidal volume, frequency and minute ventilation were determined in the hamster. Afterwards the hamster was removed from the chamber and its body temperature was measured using a Senterk BAT-12 thermometer and a Physiotemp thermocouple.

For the bromocriptine study, hamsters received subcutaneous injections of either saline or 1.0 mg/kg bromocriptine in saline (Tocris Bioscience, Ellisville, MO). This dose was used in a previous study in our laboratory (Schlenker and Schultz, 2012a). The same procedures to evaluate oxygen consumption, control of ventilation and body temperature as described for carboxirole were used in this study.

2.4 Evaluation of Body, Ovary and Uterine Weights, T4 Serum Levels, and Western Blot Analysis for D2 Receptors

At the end of the physiological studies, hamsters were weighed and deeply anesthetized. Following sacrifice, trunk blood was collected for thyroxine serum analysis. Then the brain and carotid bodies was quickly removed, frozen on dry ice, and stored at -80 degrees Celsius. Ovaries and uteri were dissected and placed in 70% ethanol and after blotting were weighed at one time.

For measurement of total thyroxine (T4) levels, the blood was spun down, the serum extracted, and frozen at -20 degrees Celsius for thyroxine determination. Thyroxine levels were measured in duplicate using a solid-phase competitive ELISA kits according to the manufacture's protocol (T4 kit Diagnostic Systems Laboratories, Inc. Webster, TX).

Tissue samples using punches from striatum, NTS and whole carotid bodies from control and PTU female hamsters were obtained as previously described (Schlenker and Schultz, 2011; 2012). The protein from brain punches (Striatum, NTS) and carotid bodies was extracted with the Radio-Immunoprecipitation Assay lysing buffer (RIPA containing 150 mM NaCl, 1.0% IGEPAL[®] CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris; pH 8.0, Sigma, USA) plus 1% complete protease inhibitor cocktail (Sigma, USA). Following ultrasonic disruption samples were centrifuged at 12,000g for 30min at 4 °C. Protein concentration in the supernatant was determined using a BCA protein assay kit (ThermoScientific, USA). The protein sample was mixed with loading buffer containing β -mercaptoethanol and heated at 100 °C for 5 min. Equal amount of protein (35 μ g) was loaded into 10% polyacrylamide gel along with molecular weight standards (PrecisionPlus Protein Standards, Bio-Rad, USA) for electrophoretic separation. Gels containing the proteins were then transferred to PVDF membrane (Immobilon-P, Millipore, USA) at 4°C. To determine D2 receptor protein levels in the three brain regions, the membranes were first incubated with blocking buffer (Odyssey Blocking Buffer, Li-Cor, USA) and then incubated overnight at 4°C with rabbit polyclonal antibody (1:4000 against a synthetic peptide AARRAQELE, corresponding to amino acids 272–282 of human D2DR, Abcam, USA). Then, membranes were washed with cold PBS (Sigma, USA) containing 0.2% Tween-20 (Sigma, USA) and incubated for 1h at room temperature with a secondary goat anti-rabbit antibody (1:10000, IRDye 800CW, Li-Cor, USA). Membranes were then developed using the Odyssey infrared fluorescence imaging systems (Li-Cor, USA).

For carotid bodies, the techniques described above for running Western blots were followed, although the membrane was probed with rabbit D2 dopamine receptor antibody (1:500 against the 1–50 of the human D2DR, Santa Cruz, USA). Membranes were then washed with cold PBS (Sigma, USA) containing 0.2% Tween-20 (Sigma, USA), incubated for 1h at room temperature with goat anti-rabbit antibody (1:10000, IRDye 800CW, Li-Cor, USA) and developed using infrared fluorescence systems (Li-Cor, USA).

Following stripping of the membranes, protein loading was evaluated by probing all membranes with mouse anti- β -actin antibody (1:2500, Sigma, USA). Stripping protocol consist in 30 min incubation with the proper stripping buffer solution (NewBlot[™], Li-Cor, USA). Stripping efficacy was determined by the absence of fluorescence signal after the

incubation with the stripping buffer. Then, membranes were incubated with the anti- β -actin antibody and develop as previously described.

Analysis of the specific bands optical densities were performed using the Odyssey Infrared Fluorescence Imaging Software (Li-Cor, USA). All data were normalized using dopamine receptor protein intensities to that of β -actin. No difference in the level of expression of β -actin was found between normal and PTU samples.

2.5 Statistical Analysis

Statistical analysis of the ventilatory and oxygen consumption data consisted of two-way repeated analysis of variance (ANOVA) to test the effects of treatment (PTU versus tap water) and drug administration separately for the 2 studies: carboxirole and bromocriptine relative to the respective vehicle controls used. If the result was significant ($P < 0.05$), post hoc Holm-Sidak tests for paired and unpaired comparisons were conducted. Body weights, body-weight-corrected ovary and uterine weights, body temperature, thyroxine levels, and Western blot data for each brain region and the carotid bodies were analyzed using unpaired Student's *t* tests. If the data were skewed, we used nonparametric tests, the Mann-Whitney test (for unpaired comparisons) or the Wilcoxon sign rank test (for paired comparisons). Data were analyzed using IBM SPSS Software Version 19.0. All data are presented as means and standard error of the mean.

3.0 Results

3.1 Body Weights, Organ Weights and T4 Serum Levels

At the conclusion of the studies, body weights (BW) of hypothyroid hamsters were significantly lower (150.6 ± 3.0 grams) compared with those of euthyroid hamsters (163.4 ± 4.2 grams, $P = 0.03$). By contrast, uterine (hypothyroid: 4.60 ± 0.12 versus euthyroid: 4.58 ± 0.17 grams/BW) and ovarian weights (hypothyroid: 0.32 ± 0.03 versus euthyroid: 0.35 ± 0.05 grams/BW) were similar the two groups. Serum T4 levels were significantly lower in the hypothyroid group (0.115 ± 0.112 $\mu\text{g/dl}$) compared to the euthyroid group (1.64 ± 0.37 $\mu\text{g/dl}$, $P < 0.01$).

3.2 Effects of PTU and Drug Treatments on Body Temperatures and Oxygen Consumption

With saline treatment, but not DMSO vehicle treatment, body temperature was lower in the hypothyroid relative to euthyroid hamsters (Table 1, $P < 0.05$). Relative to saline, bromocriptine significantly decreased body temperature in the euthyroid hamsters ($P < 0.01$), but increased it in the hypothyroid hamsters ($P < 0.05$) as shown by a significant interaction ($F_{1, 17} = 12.36$, $P < 0.01$). Carboxirole did not affect body temperatures. Bromocriptine relative to saline increased oxygen consumption in the hypothyroid hamsters ($P = 0.02$), but had no effect on oxygen consumption in the euthyroid hamsters. Carboxirole had no effect on oxygen consumption in either group.

3.3 Effects of Thyroid Status on D2 Receptor Protein Levels

Western blot results indicated that D2 receptors were expressed in the striatum, NTS and carotid bodies of hamsters from both groups (Figure 1). However, no difference between

euthyroid and hypothyroid female hamsters was noted in the relative protein levels of the D2 receptor in any region.

3.4 Effects of Bromocriptine on Ventilation

Air—There was no difference in baseline (air) minute ventilation between hypothyroid (n=9) and euthyroid (n=10) groups, although frequency of breathing was lower in the hypothyroid hamsters ($P<0.05$). During exposure to baseline air, bromocriptine relative to saline had no effect on minute ventilation in euthyroid animals (Figure 2), while surprisingly, bromocriptine increased minute ventilation ($P=0.01$) in the hypothyroid females (interaction: $F_{1, 17} = 4.76$, $P=0.04$). Although tidal volume (Figure 2B) was not affected by bromocriptine treatment in either group, bromocriptine significantly decreased frequency of breathing (Figure 2C) in the euthyroid group ($P<0.02$), but increased it in the hypothyroid group ($P<0.02$, $F_{1, 17} = 22.2$, $P<0.001$).

Hypoxia—With saline treatment the increase in minute ventilation during exposure to hypoxia was similar between the two groups. Following bromocriptine administration, the hypoxia-induced increase in ventilation in the hypothyroid hamsters was greater than that of the euthyroid hamsters ($P=0.04$). The greater increase in minute ventilation was attributed to an increase of tidal volume in the bromocriptine-treated hypothyroid hamsters ($P<0.01$) without affecting tidal volume in euthyroid hamsters. Compared to saline treatment, bromocriptine had no effect on frequency of breathing in either group. Hypoxic responsiveness, the ratio of minute ventilation during exposure to hypoxia divided to the minute ventilation during baseline air, showed a significant effect of drug treatment ($F_{1, 17} = 7.27$, $P=0.015$). Specifically, bromocriptine depressed hypoxic responsiveness in the hypothyroid group ($P<0.05$), while having no effect on hypoxic responsiveness in the euthyroid group (Figure 4A).

Post-hypoxia—Following exposure to hypoxia minute ventilation and tidal volume returned to pre-hypoxic levels in both vehicle treated groups and did not differ between groups. However, with bromocriptine treatment minute ventilation remained elevated in the hypothyroid animals ($P=0.045$), but not in the euthyroid hamsters (Interaction, $F_{1, 17} = 6.03$, $P=0.025$). As a consequence, following hypoxic exposure minute ventilation was greater in the bromocriptine-treated hypothyroid hamsters compared to bromocriptine-treated euthyroid hamsters ($p<0.01$). With bromocriptine treatment, tidal volume was greater, but breathing frequency was slower ($P<0.01$) in euthyroid compared to hypothyroid animals ($P<0.04$). Bromocriptine compared to saline markedly elevated frequency of breathing ($P<0.03$) in the hypothyroid hamsters without affecting tidal volume, accounting for the increased post-hypoxic minute ventilation in this group. Post hypoxic responsiveness (minute ventilation following hypoxic exposure divided by the baseline air value) displayed an overall effect of drug treatment ($F_{1, 17} = 7.27$, $P=0.015$) but there was not different between the two groups (Figure 6).

Overall, in air-exposed hypothyroid hamsters, bromocriptine increased body temperature and oxygen consumption. In this group bromocriptine relative to saline also stimulated ventilation before, during and after exposure to hypoxia. By contrast, bromocriptine had

either no or opposite effects on these parameters in the euthyroid animals. Representative tracings of ventilatory responses to bromocriptine of both groups to air, hypoxia and post-hypoxia are shown in Figure 3.

3.5 Effects of Carmoxirole on Ventilation

Air—With vehicle treatment during baseline air exposure, there were no differences in tidal volume, frequency or minute ventilation (Figure 4) between the hypothyroid and euthyroid hamsters. While hamsters were exposed to baseline air, carmoxirole did not affect minute ventilation or tidal volume in either group. In contrast, relative to vehicle, carmoxirole decreased frequency of breathing the euthyroid hamsters ($P=0.01$, interaction $F_{1, 17}=17$, $P=0.013$). Consequently, breathing frequency was faster in carmoxirole-treated hypothyroid animals as compared to euthyroid hamsters.

Hypoxia—Hypothyroid hamsters treated with carmoxirole increased their minute ventilation during exposure to hypoxia more than did the euthyroid hamsters ($P<0.013$, interaction $F_{1, 17}=8.5$, $P=0.01$). Relative to vehicle treatment, the increase in frequency of breathing in response to hypoxia was less with carmoxirole treatment in the euthyroid hamsters ($P<0.03$), while carmoxirole had no effect on the frequency response to hypoxia in the hypothyroid animals. The enhanced minute ventilation response to hypoxia in carmoxirole treated hypothyroid hamsters was due to an increase in tidal volume relative to that of carmoxirole treated euthyroid hamsters ($P=0.01$). Hypoxic responsiveness was not different between the two groups nor affected by carmoxirole treatment (Figure 4A).

Post-hypoxia—Following exposure to hypoxia minute ventilation was lower in the vehicle-treated hypothyroid hamsters compared to the vehicle-treated euthyroid animals ($P=0.04$). Relative to vehicle, carmoxirole decreased post-hypoxic minute ventilation in the euthyroid hamsters ($P<0.001$). As a consequence, post-hypoxic minute ventilation in carmoxirole-treated hypothyroid hamsters was greater than that of carmoxirole-treated euthyroid hamsters ($P<0.05$). With carmoxirole, relative to vehicle treatment, frequency of breathing slowed significantly in the euthyroid hamsters post hypoxia ($P<0.01$), but remained elevated in the hypothyroid hamsters ($P=0.005$, Interaction, $F_{1, 17}=78.8$, $P<0.0001$). Interestingly, tidal volume was smaller in carmoxirole-treated hypothyroid hamsters compared to that of the euthyroid animals ($P=0.01$). Moreover, following carmoxirole treatment, minute ventilation post hypoxic responsiveness was greater in the hypothyroid hamster relative to the euthyroid hamsters (Figure 6B, $P<0.05$).

Overall, carmoxirole had no effect on body temperature or oxygen consumption in either group. This contrasts with the centrally acting effects of bromocriptine reported in this study. Relative to vehicle treatment carmoxirole consistently (during exposure to air, hypoxia, and following hypoxia) decreased frequency of breathing in the euthyroid group, but increased frequency of breathing and decreased tidal volume post-hypoxia in the hypothyroid hamsters. Representative tracings of ventilatory responses of both groups treated with carmoxirole and exposed to air, hypoxia and post-hypoxic air are shown in Figure 5.

4.0 Discussion

Relative to euthyroid female hamsters, hypothyroid animals showed lower body weights, but similarities in body weight-normalized uterine and ovarian weights, and in the expression of D2 receptors in the striatum, NTS and carotid bodies. The lower T4 serum levels and body weights were indicators of hypothyroidism. However, oxygen consumption and ventilation during baseline air exposure was comparable between the two groups. Unlike peripherally acting carboxirole that did not affect body temperature, the centrally acting bromocriptine affected body temperatures in both groups in disparate ways and increased oxygen consumption in the hypothyroid group. While there were few ventilatory changes that could be attributed to hypothyroidism alone as seen with the vehicle data, there were significant effects of D2 receptor stimulation on ventilation during exposure to air, hypoxia and air following hypoxic exposure due to hypothyroidism.

4.1 Expression of D2 receptors

Unlike previous studies in comparably aged male hamsters treated with PTU for 5 months that showed significantly higher levels of D2 receptors in the NTS but not in the striatum or carotid bodies relative to euthyroid males (Schlenker and Schultz, 2012a), no differences in D2 receptor expression in this region were noted in female hamsters treated with PTU for 5 months relative to euthyroid females. These results suggest that there may be a sex difference in the effects of hypothyroidism on the expression of D2 receptors in the NTS.

4.2 Effects of hypothyroidism on reproductive organs

Hypothyroidism has been shown to influence reproduction in females that may be dependent on the severity and length of time of the hypothyroid treatment. This could possibly affect ventilation by changes in sex steroid hormone levels such as estradiol and progesterone (Wenninger et al., 2009). Moreover, the length and severity of hypothyroidism needs to be considered. For example, in a short term study in female rats given 1gram/L PTU in drinking water (about 25 times the amount used in the present study) for 6 to 9 days, Hapon and colleagues (Hapon et al., 2010) found small changes in hormone levels (prolactin, growth hormone, insulin growth factor -1, and estradiol) only at specific times of the estrus cycle. No effects were noted on the corpus lutea or number of oocytes. When Vriend and colleagues (Vriend et al., 1987) administered 0.4% thiourea in drinking water to female hamsters for 10 weeks the investigators noted that animals had irregular estrus cycles, decreased uterine weights, lower number of oocytes, and decreased serum estradiol levels relative to euthyroid hamsters. In the present study uterine and ovarian weights were comparable between the hypothyroid and euthyroid groups, but the concentration of PTU in the drinking water was 10 times less than the thiourea in the Vriend study. Although the current set of studies did not take into consideration serum hormone levels or estrus cycles of the female hamsters no effects of 0.04% PTU on the female reproductive organs weights were observed. A more systematic study including measurement of estradiol and progesterone and correlations with ventilatory parameters is needed to determine if there were effects of hypothyroidism on hormone levels that may influence control of breathing in female hamsters.

4.3 Effects of hypothyroidism on D2 receptor modulated ventilation and metabolism

A recent study investigated the effects of bromocriptine on ventilation in 7 month old hypothyroid (who had been hypothyroid for 5 months) and age-matched euthyroid male hamsters (Schlenker and Schultz, 2012a). Ventilation in air was depressed in both groups of animals following administration of bromocriptine. This contrasts with the results in the present study where during air exposure bromocriptine stimulated minute ventilation and frequency of breathing, oxygen consumption and increased body temperature in the hypothyroid female hamsters, but decreased body temperature and oxygen consumption, but not ventilation in euthyroid female hamsters. One potential mechanism by which bromocriptine could indirectly stimulate ventilation during air exposure is by increasing metabolism and body temperature in the hypothyroid females. This bromocriptine-induced increase in metabolism and body temperature in hypothyroid female hamsters is atypical from the hypothermic effect of bromocriptine previously reported in rodents and human (Nava et al., 2000; Schlenker and Schultz, 2012b; Schwartz and Erk, 2004; Tsuchiya et al., 2012) and noted in euthyroid hamsters in the present study. The effects of D2 receptor agonists on metabolism during exposure of animals to hypoxia has not been studied. Whether changes in oxygen consumption may explain the differences in ventilatory responsiveness observed in the hypothyroid and euthyroid female hamsters in the present study needs to be examine.

In contrast, during baseline air exposure carboxirole did not affect body temperature or oxygen consumption in either group but it decreased breathing frequency in euthyroid hamsters. In hypothyroid hamsters the stimulatory effects of carboxirole on frequency of breathing after hypoxia suggests a peripherally mediated effect of D2 receptors, possibly within the carotid body. Thus following hypoxic exposure, both central nervous system and peripherally located D2 receptors are involved in regulating breathing in hypothyroid female hamsters.

4.4 How D2 receptors can become excitatory

The present study indicated that although there were no effects of PTU treatment on levels of D2 receptors in brain regions and carotid bodies, in female hypothyroid hamsters, D2 receptor stimulation with bromocriptine increased ventilation relative to female euthyroid hamsters during air exposure and markedly following exposure of animals to hypoxia. Several possible mechanisms may be responsible for these observations. One mechanism may be that in hypothyroid hamsters there is a difference in dopamine D2 receptor expression in presynaptic and postsynaptic sites. One approach to evaluate this possibility is to investigate the D2 isoforms using RT-PCR method (Kendall and Senogles, 2011). Dopamine D2 receptors are expressed in 2 isoforms: D2short (D2S) and D2long (D2L). The D2S is located presynaptically and modulates production and release of dopamine (Anzalone et al., 2012). The other isoform, D2L is located postsynaptically and decreases production of cAMP and phosphorylation of DARP. Thus, alterations in the relative levels of these receptor isoforms may affect function. Preliminary results from our laboratory (Sykora, Schlenker, and Eyster) indicate that there is a reduction of D2L relative to D2S isoforms in NTS of hypothyroid female hamsters relative to age matched euthyroid females. Interestingly, no effects of hypothyroidism on D2S or D2L levels were found in the

striatum, hypoglossal nuclei, or hippocampus, but the ratio of D2L to D2S was increased in the hypoglossal nuclei but decreased in the hippocampus. These results suggest regional differences in the expression of these two isoforms. To determine the functional consequences of these findings, D2L receptors could be knocked down in the NTS of euthyroid hamsters to see if they mimic the responses of hypothyroid animals to bromocriptine microinjections.

Another mechanism that may result in stimulation of breathing in hypothyroid hamsters following exposure to hypoxia is greater internalization of D2 receptors than that seen in euthyroid hamsters. During exposure to hypoxia, if there were greater internalization of D2 receptors in the hypothyroid hamsters than euthyroid hamsters, the dopamine may increasingly act on D1 receptors (Genedani et al., 2010; Li et al., 2012; Tirotta et al., 2008). Unpublished results indicate that D1 receptors are located on the NTS and carotid bodies of euthyroid and hypothyroid female hamsters in comparable levels. Investigations are underway to test the hypothesis that increased internalization of D2 receptors following hypoxic exposures may be a mechanism to explain the differential responses noted in this and in previous studies.

Although not investigated specifically in hypothyroidism, D2 receptor's ability to maintain a negative tone can be modulated epigenetically by the regulation of molecules such as prostate apoptosis response-4 that modulate expression and function of D2 receptors (Park et al., 2005). Normal binding of Par-4 to D2 receptors has been shown to be decreased in stress and depression (Moriya and Sobhani, 2013) due to elevated levels of intracellular Ca^{2+} . If hypothyroidism decreased Par-4 levels in female hamsters, this may influence the stimulatory effects D2 receptor agonists and/or hypoxia have on breathing. Future studies are needed to investigate these possibilities.

4.5 Conclusions

In summary, D2 receptors are expressed to the same extent in striatum, NTS and carotid bodies of female euthyroid and hypothyroid hamsters, but modulation of breathing is affected differently by stimulating D2 receptors in these two groups of animals. Thus, hypothyroidism must affect the function of these receptors differently in hypothyroid and euthyroid animals. Moreover, comparing the results of this study with those in age and treatment- matched males suggest that there may be a sexual dimorphism in the function of D2 receptors in regulation of breathing. Future studies will systematically examine this possibility and the underlying mechanism responsible for these findings.

Acknowledgements

This study was supported by a Sanford School of Medicine Internal grant. We thank Dr. Kathleen Eyster for her help with the D2 isoform study. The Genomics Core is supported by NIGMS #P20GM103443

References

- Anzalone A, Lizardi-Ortiz JE, Ramos M, De Mei C, Hopf FW, Iaccarino C, Halbout B, Jacobsen J, Kinoshita C, Welter M, Caron MG, Bonci A, Sulzer D, Borrelli E. Dual Control of Dopamine Synthesis and Release by Presynaptic and Postsynaptic Dopamine D2 Receptors. *J Neurosci*. 2012; 32:9023–9034. [PubMed: 22745501]

- Bairam A, Carroll JL. Neurotransmitters in carotid body development. *Respir Physiol Neurobiol.* 2005; 149:217–232. [PubMed: 15919246]
- Beaulieu J-M, Gainetdinov R. The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev.* 2011; 63:182–217. [PubMed: 21303898]
- Braverman, L.; Utiger, R. Werner & Ingbar's the thyroid: a fundamental and clinical text. 9th ed.. Philadelphia: Lippincott, Williams and Wilkins; 2005.
- Burton MD, Kazemi H. Neurotransmitters in central respiratory control. *Respiration Physiology.* 2000; 122:111–121. [PubMed: 10967338]
- Canaris GJ, Manowitz NR, Mayor G, Ridgway EC. The Colorado thyroid tisease prevalence study. *Arch Intern Med.* 2000; 160:526–534. [PubMed: 10695693]
- Chenuel BJ, Smith CA, Henderson KS, Dempsey JA. Increased propensity for apnea via dopamine-induced carotid body inhibition in sleeping dogs. *J Appl Physiol.* 2005; 98:1732–1739. [PubMed: 15591296]
- Dempsey JA, Smith CA, Blain GM, Xie A, Gong Y, Teodorescu M. Role of central/peripheral chemoreceptors and their interdependence in the pathophysiology of sleep apnea. *Advances in experimental medicine and biology.* 2012; 758:343–349. [PubMed: 23080181]
- Empson M, Flood V, Ma G, Eastman CJ, Mitchell P. Prevalence of thyroid disease in an older Australian population. *Intern Med J.* 2007; 37:448–455. [PubMed: 17547723]
- Evans KC, Shea SA, Saykin AJ. Functional MRI localisation of central nervous system regions associated with volitional inspiration in humans. *J Physiol.* 1999; 520:383–392. [PubMed: 10523407]
- Genedani S, Carone C, Guidolin D, Filaferro M, Marcellino D, Fuxe K, Agnati LF. Differential sensitivity of A2A and especially D2 receptor trafficking to cocaine compared with lipid rafts in cotransfected CHO cell lines. Novel actions of cocaine independent of the DA transporter. *J Mol Neurosci.* 2010; 41:347–357. [PubMed: 20143275]
- Hapon M, Gamarra-Luques C, Jahn G. Short term hypothyroidism affects ovarian function in the cycling rat. *Repro Bio Endocrinol.* 2010; 8:14.
- Hyde TM, Knable MB, Murray AM. Distribution of dopamine D1–D4 receptor subtypes in human dorsal vagal complex. *Synapse.* 1996; 24:224–232. [PubMed: 8923662]
- Kansagra SM, McCudden CR, Willis MS. The challenges and complexities of thyroid hormone replacement. *Lab Medicine.* 2010; 41:338–348.
- Kendall RT, Senogles SE. Isoform-specific uncoupling of the D2 dopamine receptors subtypes. *Neuropharmacology.* 2011; 60:336–342. [PubMed: 20888845]
- Kumar, P.; Prabhakar, NR. Peripheral Chemoreceptors: Function and Plasticity of the Carotid Body, *Comprehensive Physiology.* John Wiley & Sons, Inc.; 2011. p. 141-219.
- Lakshmi V, Vaney N, Madhu SV. Effect of thyroxine therapy on autonomic status in hypothyroid patients. *Indian J Physiol Pharmacol.* 2009; 53:219–226. [PubMed: 20329368]
- Lalley PM, Mifflin SW. Opposing effects on the phrenic motor pathway attributed to dopamine-D1 and -D3/D2 receptor activation. *Respir Physiol Neurobiol.* 2012; 181:183–193. [PubMed: 22465544]
- Li Y, Roy BD, Wang W, Zhang L, Zhang L, Sampson SB, Yang Y, Lin D-T. Identification of two functionally distinct endosomal recycling pathways for dopamine D2 receptor. *J Neurosci.* 2012; 32:7178–7190. [PubMed: 22623662]
- Lucas A, Julián M, Cantón A, Castell C, Casamitjana R, Martínez-Cáceres E, Granada M. Undiagnosed thyroid dysfunction, thyroid antibodies, and iodine excretion in a Mediterranean population. *Endocrine.* 2010; 38:391–396. [PubMed: 20972723]
- Mainenti MRM, Teixeira PFS, Oliveira FP, Vaisman M. Effect of hormone replacement on exercise cardiopulmonary reserve and recovery performance in subclinical hypothyroidism. *Braz J Med Bio Res.* 2010; 43:1095–1101. [PubMed: 21088807]
- Moriam S, Sobhani ME. Epigenetic Effect of Chronic Stress on Dopamine Signaling and Depression. *Genetics & Epigenetics.* 2013; 5:11–16. [PubMed: 25512703]
- Nattie E, Shi J, Li A. Bicuculline dialysis in the retrotrapezoid nucleus (RTN) region stimulates breathing in the awake rat. *Respiration Physiology.* 2001; 124:179–193. [PubMed: 11173073]

- Nava F, Carta G, Gessa GL. Permissive role of dopamine D2 receptors in the hypothermia induced by 9-Tetrahydrocannabinol in rats. *Pharmacol Biochem Behav.* 2000; 66:183–187. [PubMed: 10837859]
- Nielsen AM, Bisgard GE. Differential effects on phrenic output of two dopamine agonists, apomorphine and bromocriptine. *Eur J Pharmacol.* 1984; 106:69–78. [PubMed: 6529974]
- Nobrega JN, Richter A, Tozman N, Jiwa D, Loscher W. Quantitative autoradiography reveals regionally selective changes in dopamine D1 and D2 receptor binding in the genetically dystonic hamster. *Neuroscience.* 1996; 71:927–936. [PubMed: 8684623]
- O'Halloran KD, Janssen PL, Bisgard GE. Dopaminergic modulation of respiratory motor output in peripherally chemodenervated goats. *J Appl Physiol.* 1998; 85:946–954. [PubMed: 9729568]
- Park SK, Nguyen MD, Fischer A, Luke MP-S, Affar EB, Dieffenbach PB, Tseng H-C, Shi Y, Tsai L-H. Par-4 Links Dopamine Signaling and Depression. *Cell.* 2005; 122:275–287. [PubMed: 16051151]
- Ramirez J-M, Garcia Iii AJ, Anderson TM, Koschnitzky JE, Peng Y-J, Kumar GK, Prabhakar NR. Central and peripheral factors contributing to obstructive sleep apneas. *Respiratory Physiology & Neurobiology.* 2013; 189:344–353. [PubMed: 23770311]
- Reddy MK, Patel KP, Schultz HD. Differential role of the paraventricular nucleus of the hypothalamus in modulating the sympathoexcitatory component of peripheral and central chemoreflexes. *Am J Physiol Regul Integr Comp Physiol.* 2005; 289:R789–R797. [PubMed: 15919733]
- Schlenker E, Means R, Burbach J. Naloxone stimulates oxygen consumption but not ventilation in hypothyroid hamsters. *Physiol Behav.* 1994; 56:635–638. [PubMed: 7800724]
- Schlenker E, Schultz H. Hypothyroidism stimulates D2 receptor-mediated breathing in response to acute hypoxia and alters D2 receptors levels in carotid bodies and brain. *Respir Physiol Neurobio.* 2012a; 180:69–78.
- Schlenker EH. An evaluation of ventilation in dystrophic Syrian hamsters. *J Applied Physiol.* 1984; 56:914–921. [PubMed: 6725069]
- Schlenker EH. In hamsters dopamine D2 receptors affect ventilation during and following intermittent hypoxia. *Comp Biochem Physiol- Part A: Mol Int Physiol.* 2007; 148:674–680.
- Schlenker EH, Schultz HD. Hypothyroidism attenuates SCH 23390-mediated depression of breathing and decreases D1 receptor expression in carotid bodies, PVN and striatum of hamsters. *Brain Res.* 2011; 1405:40–51. [PubMed: 21669406]
- Schlenker EH, Schultz HD. Hypothyroidism stimulates D2 receptor-mediated breathing in response to acute hypoxia and alters D2 receptors levels in carotid bodies and brain. *Respir Physiol Neurobio.* 2012b; 180:69–78.
- Schwartz PJ, Erk SD. Regulation of central dopamine-2 receptor sensitivity by a proportional control thermostat in humans. *Psychiatry Res.* 2004; 127:19–26. [PubMed: 15261701]
- Sykora C, Amor M, Schlenker E. Age and hypothyroidism affect dopamine modulation of breathing and D2 receptor levels. *Respir Physiol Neurobio.* 2013; 185:257–264.
- Tirotta E, Fontaine V, Picetti R, Lombardi M, Samad TA, Oulad-Abdelghani M, Edwards R, Borrelli E. Signaling by dopamine regulates D2 receptors trafficking at the membrane. *Cell Cycle.* 2008; 7:2241–2248. [PubMed: 18635969]
- Tsuchiya N, Iwase M, Izumizaki M, Homma I. Dopaminergic modulation of exercise hyperpnoea via D2 receptors in mice. *Exper Physiol.* 2012; 97:228–238. [PubMed: 22041981]
- Varney AA, Schlenker EH. Thyroid status affects 5-HT_{2A} receptor modulation of breathing before, during, and following exposure of hamsters to acute intermittent hypoxia. *Am J Physiol Regul Integr Comp Physiol.* 2007; 293:R2070–R2080. [PubMed: 17855493]
- Vriend J, Bertalanffy FD, Ralcewicz TA. The effects of melatonin and hypothyroidism on estradiol and gonadotropin levels in female Syrian hamsters. *Bio Reprod.* 1987; 36:719–728. [PubMed: 3109508]
- Wenninger JM, Olson EB, Cotter CJ, Thomas CF, Behan M. Hypoxic and hypercapnic ventilatory responses in aging male vs. aging female rats. *J Appl Physiol.* 2009; 106:1522–1528. [PubMed: 19265066]

Figure 1.

Representative Western blot and protein levels of dopamine D2 receptors in striatum, nucleus tractus solitaries (NTS) and carotid bodies (CB) of euthyroid (black bars) and hypothyroid hamsters (white bars). Values are means and SEM of 4–6 animals per group per region.

Figure 2.

Effects of vehicle and bromocriptine on ventilation (A), tidal volume (B) and frequency of breathing (C) in euthyroid (black bars) and hypothyroid (white bars) hamsters exposed air, hypoxia and air following hypoxia (Post-Hypoxia). The asterisks denote differences between the hypothyroid (n=10) and euthyroid groups (n=9) for each exposure while the pound signs denote differences due to bromocriptine relative to vehicle treatment. Values are means and SEM.

Figure 3.

Representative traces of ventilatory responses of a euthyroid and a hypothyroid hamster treated with bromocriptine and exposed to air, hypoxia and then air following hypoxia.

Figure 4.

Effects of vehicle and carboxirole on ventilation (A), tidal volume (B) and frequency of breathing (C) in 9 euthyroid (black bars) and 10 hypothyroid (white bars) hamsters exposed air, hypoxia and air following hypoxia (Post-Hypoxia). The asterisks denote differences between the hypothyroid and euthyroid groups for each exposure while the pound signs denote differences due to carboxirole relative to vehicle. Values are means and SEM.

Euthyroid Carmoxirole

Hypothyroid Carmoxirole

Figure 5.

Representative traces of ventilatory responses of a euthyroid and a hypothyroid hamster treated with carboxirole and exposed to air, hypoxia and then air following hypoxia.

Figure 6.

Effects of vehicle, bromocriptine and carboxirole on A. hypoxic responsiveness and B. post-hypoxic responsiveness in euthyroid (black bars) and hypothyroid (white bars) hamsters. The asterisks denote significant differences of treatments effects on responses of hypothyroid and euthyroid hamsters. Values are means and SEM.

Table 1

Body Temperature (in degrees Celsius) and Oxygen Consumption (ml/min *1000/BW) Data for Hypothyroid and Euthyroid Hamsters Receiving Vehicle (saline or 5% DMSO), Bromocriptine or Carmoxirole

Body Temperature		
Treatment	Euthyroid	Hypothyroid
Vehicle (saline)	36.8 (0.2)	35.8 (0.4) *
Bromocriptine	35.1 (0.4) #	36.3 (0.2) #
Vehicle (DMSO)	36.6 (0.2)	36.0 (0.3)
Carmoxirole	36.0 (0.2)	35.9 (0.2)
Oxygen Consumption		
Treatment	Euthyroid	Hypothyroid
Vehicle (saline)	18.1 (1.6)	16.9 (1.3)
Bromocriptine	18.9 (2.0)	21.2 (4.9) #*
Vehicle (DMSO)	20.2 (1.4)	20.0 (1.9)
Carmoxirole	22.4 (1.6)	21.9 (2.1)

Because 2 different vehicles were used, bromocriptine data were compared to the saline and the carmoxirole data were compared to the 5% DMSO data.

Asterisks denote significant effects of group (hypothyroid relative to euthyroid);

the # denotes significant effects of bromocriptine relative to saline.