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The Ventilatory Response to Normoxic Hypercapnia in Male Pet-1 Knockout Mice and the Influence of Female Sex Hormones

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Abstract

THE VENTILATORY RESPONSE TO NORMOXIC HYPERCAPNIA IN MALE PET-1 KNOCKOUT MICE AND THE INFLUENCE OF FEMALE SEX HORMONES.

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Purpose: To determine if the deficiency in the hypercapnic ventilatory response as a result of 70% loss of serotonergic neurons in male Pet-1 knockout mice and the sparing of the female Pet-1 knockout mice can been accounted for and corrected by supplementation with estrogen and progesterone.

Methods: Using whole-body flow-through plethysmography, 5 male Pet-1 -/- mice and 5 wild type mice were exposed to room air, 3%, 5%, 7%, and 10% CO_2 and V_E measured. All mice were then treated with oral 17β-estradiol (2500 nM) for 48 hours followed by a SC injection of progesterone (2 mg/kg). One hour after injection all plethysmography studies were repeated.

Results: Before treatment Pet-1 knockout mice showed a trend towards decreased V_E at room air, 3%, and 7% CO_2, with statistical significance at 5% CO_2 ($p < 0.05$), and an identical V_E only at 10% CO_2. Absolute changes in V_E after treatment were insignificant; the differences in V_E between genotypes achieved significance at control, 3%, and 5% CO_2 ($p < 0.05$) while now V_E at 7% and 10% CO_2 exposures were identical ($p < 0.05$).

Conclusion: The partial recovery of the male Pet-1 knockout phenotype provides evidence that the remaining serotonergic neurons can recover some chemoreceptor function under the influence of female sex hormones, which in turn may partially explain the female Pet-1 knockout phenotype.

Thank you to all the members of the Richerson lab for their assistance, advice, and knowledge throughout this process. Specific thanks to John Sayward for his computer programming skills and to Matthew Hodges for his role in the plethysmography design, troubleshooting, and experimental assistance.

To Dr. George Richerson, whose tireless, rigorous, and ethical pursuit of the truth represents the best of the human intellectual spirit and is an inspiration to all who work in his lab.

Table of Contents

Introduction

As a neurotransmitter and a neural system, serotonin has been implicated in a wide range of functions and disorders. These include the regulation of sleep, aggression, anxiety, and clinical disorders such as depression, Obsessive Compulsive Disorder and SIDS. While there is much clinical, pharmaceutical, and physiologic evidence to support serotonin's role in these various functions, little attention has been paid to a unifying theory of the serotonin system that could account for these diverse roles. Given its small, centrally located nuclei and its extensive connections throughout the CNS it is not unreasonable to assume that some primary function could provide the key to its multi-faceted phenotype.

Richerson has recently proposed central chemoreception as such a unifying function for the serotonin system that takes into account some of primary properties of serotonin neurons as well as provides an explanation for its role in these diverse disorders (1).

Chemoreception is the "detection" component of the fundamental homeostatic mechanism that is necessary to maintain a body pH between tight physiologic boundaries. The primary determinant of pH in humans is carbon dioxide, through its stochiometric and enzymatic conversion to bicarbonate and a proton ($CO_2 + H_2O \rightarrow H^+ + HCO_3^-$). Therefore, candidate chemoreceptors must be able to respond to changes in pH by initiating appropriate physiologic defense mechanisms. In the case of pH, the most important acute response is the maintenance of P_{CO2} by the control of lung ventilation.

The first central chemoreceptors were located by focal acidosis of the cat medulla, and an increase in ventilation was in seen on the ventrolateral surface (VLMS) (2). This

is the location for the nuclei of multiple types of neurotransmitters, including serotonin, whose medullary groups also include the raphe pallidus, raphe magnus, and raphe obscurus (3). These serotonergic projections influence cardiovascular control, autonomic output, motor control, and pain processing (4-6). They also have a well-described effect on breathing. Respiratory nuclei including the nucleus ambiguous, nucleus tractus solitarius, phrenic motor nuclei, hypoglossal nuclei, and the preBotzinger complex contain serotonergic terminals (7-12). Exogenous application of serotonin analogues stimulates respiration in rats and cats (13, 14). This effect is even more pronounced after general anesthetic, where respiratory depression or apneusis can be reversed with serotonin agonists (12, 15). Electrical stimulation of the raphe pallidus or glutamatergic stimulation of the medullary raphe both increase phrenic nerve output (16, 17). As would be expected, selective ablation of serotonergic nuclei leads to hypoventilation (18). In sum, the primary respiratory effect of serotonin is a stimulation of breathing, thus, if it could be shown that these neurons are chemosensitive, the hypothesis that serotonin neurons in the VLMS are responsible for pH homeostasis would be an attractive one.

The lines of evidence that indicate serotonin neurons have the necessary "detection" properties required for central chemoreception can be broken down into broad categories - (1) in vitro work with single cell culture, (2) specialized anatomic considerations, (3) in vivo studies with focal inhibition or destruction of serotonin neurons, and most recently (4) genetic manipulation of the serotonin system.

In vitro

The first clue that serotonergic neurons were involved in chemoreception came from rat brain slices, where a subset of neurons in the medullary raphe were found to be highly stimulated by changes in P_{CO2} from 5% to 9% and inhibited by change to a P_{CO2} of 3% (19). Another group of neurons showed an opposite response – they were inhibited by acidosis and stimulated by alkalosis. Of these subsets, all carbon-dioxide stimulated neurons were serotonergic, and none of the carbon-dioxide inhibited were. In cell culture experiments it was determined that 75-90% of all serotonin neurons in the medullary raphe respond to CO_2 with an average response of 300% increase in firing frequency (20-22). The channel responsible for this sensitivity appears to be a slow afterhyperpolarixation calcium-activated cation current (23).

Anatomic Specialization

A striking structure-function relation exists for the serotonergic neurons in the brainstem. Confocal studies show that serotonergic neurons wrap closely along side the main arteries of the brain stem – the basilar artery and its penetrating branches – with dendrites that enwrap the vessels and come with half of a micrometer from the arterial blood (24). Teleologically, this is the ideal location for central chemoreceptors, as the basilar artery contains blood with a P_{CO2} directly from the lung, reflecting gas exchange before it is altered by brain metabolism. This anatomical specialization has not been described in other putative chemoreceptors.

In vivo

In whole animal studies there is also evidence that serotonergic neurons contribute to the hypercapnic response. After hypercapnic exposure, *C-fos* staining increases in CO_2 activated neurons – these neurons in the medullary raphe are immunoreactive for 5-HT and tryptophan hydroxylase (25, 26). In awake, normally behaving cats, a subset of serotonergic neurons increases their firing rate in response to exposure to CO_2 (27). Two experiments inducing focal acidosis in the medullary raphe – by application of acetazolamide or by microdialysis of high CO_2 solution – both showed an increase in ventilation (28, 29). Similar finding have been achieved in goats, where focal acidosis in the medulla increases respiratory output (30, 31).

Experiments to inhibit or destroy serotonin neurons in the medullary raphe have also shown the expected results. When neonatal rats are treated with 5,7-DHT, which destroys serotonin neurons, in adulthood they have elevated P_{CO2} levels at baseline and a decreased ventilatory response to CO_2 (18). Focal lesions of the serotonin system in adult rats with 8-OH-DPAT or antibody conjugated to the toxin saporin directed at the 5-HT transporter cause a decrease in the ventilatory response to carbon dioxide (32, 33).

Genetic

Most recently, the ability to manipulate the serotonin system through genetic knockouts eliminates the need for invasive or chemical manipulations (34). Due to the high interest in the serotonin system for the study of behavioral and psychiatric systems, many variant knockout mice have been developed. One knockout generated is deficient in Pet-1, an ETS domain transcription factor critical for the normal development of

serotonin neurons. Mice deficient in Pet-1 lack 70% of their serotonin neurons in all serotonergic nuclei, with no detectable deficiencies in other neurotransmitter systems or brain architecture. The remaining serotonin neurons have dramatically reduced TPH and SERT mRNA expression. These deficiencies result in only 10-15% of wild-type serotonergic fibers and quantitative levels of serotonin throughout the brain. Behaviorally these mice are more aggressive than their wild-type kindred, but have normal development, motor function and learning, reproduction, and life expectancy in adulthood, although 30% of them die in the first week of life from unknown causes (35). If the serotonin system was involved in chemoreception, Pet-1 knockout mice might be expected to have a deficient response to hypercapnia if peripheral chemoreceptors and the remaining serotonin neurons were unable to compensate sufficiently.

Indeed, recent studies with normoxic hypercapnic challenges have shown that Pet-1 knockout mice do not have a normal response [Fig 1 (a)] (36). This deficit appears to be mild, but when the results are sorted by the sex of the mice, a striking picture emerges, where the male mice have a clearly deficient response [Figure 1 (b)] and the female response is essentially normal [Figure 1 (c)]. This intriguing result suggests that some factor or factors are responsible for protecting the female mice from the knockout phenotype.

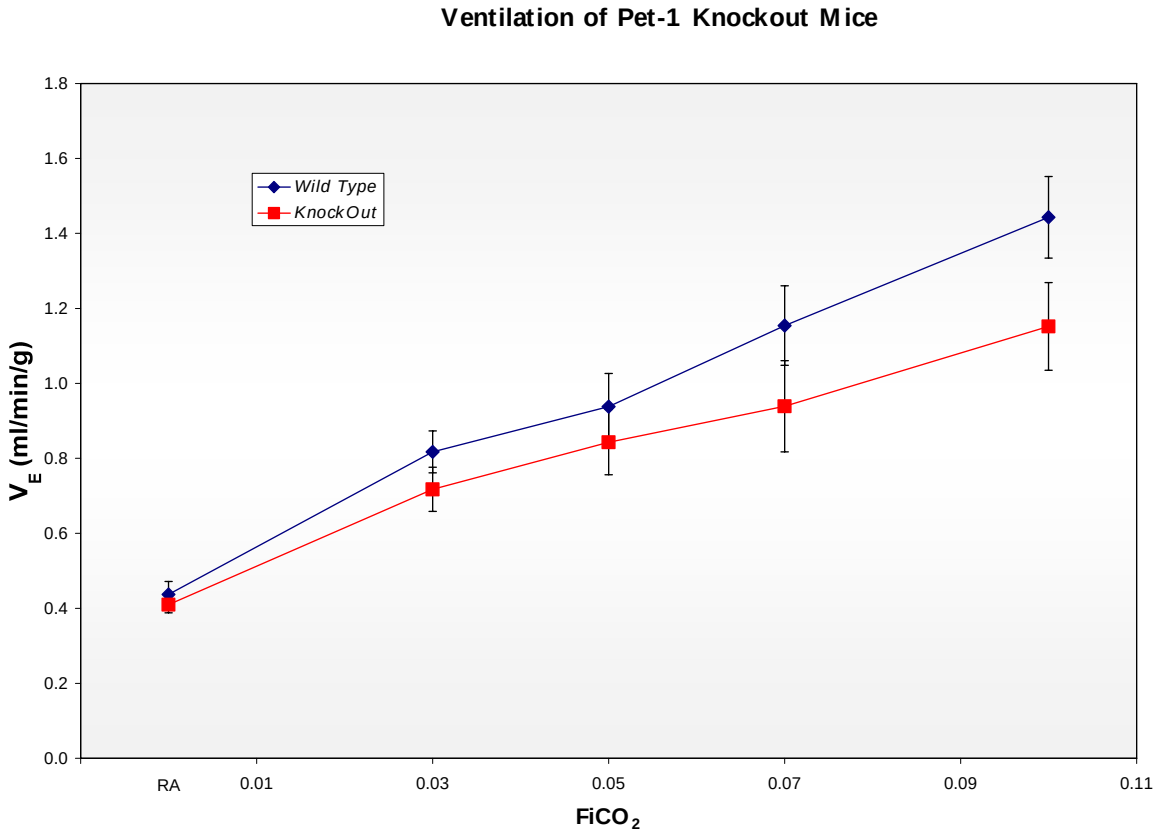

Figure 1 (a)

The most obvious source of sex differences is the influence of the female sex hormones estrogen and progesterone, especially since there is a well documented stimulatory effect of female sex hormones upon breathing, primarily progesterone, in humans and other species (37, 38).

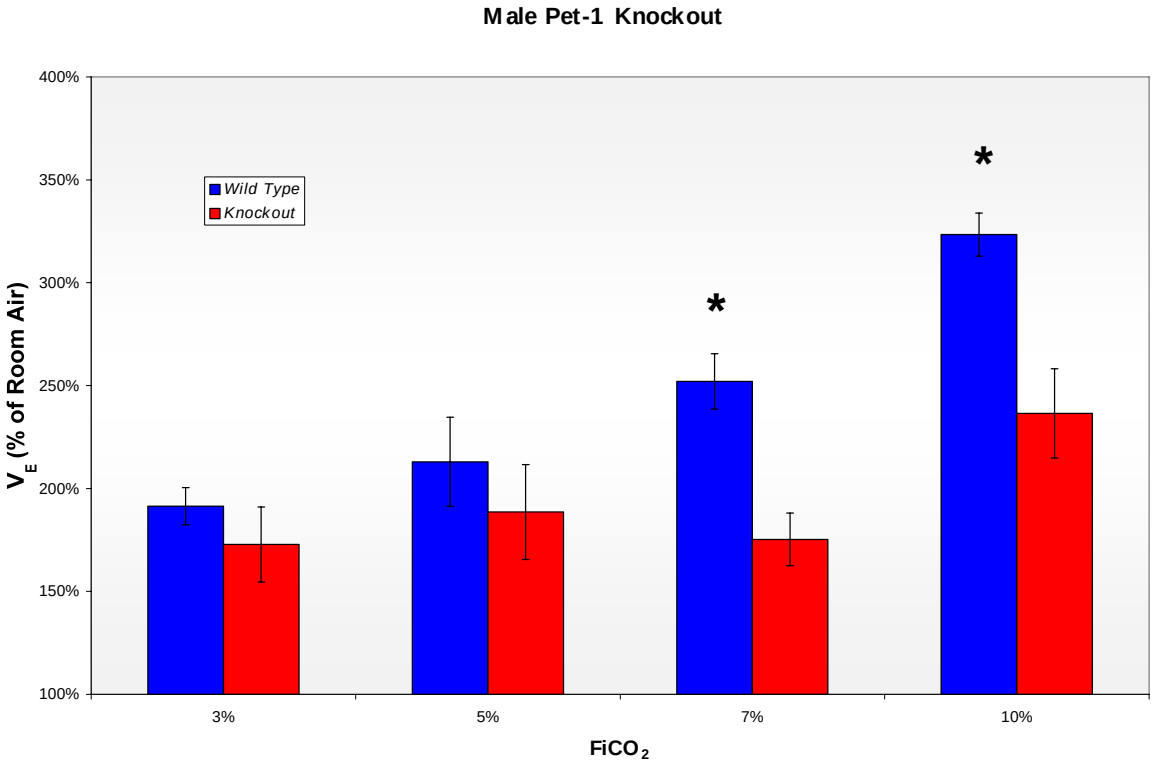

Figure 1 (b)

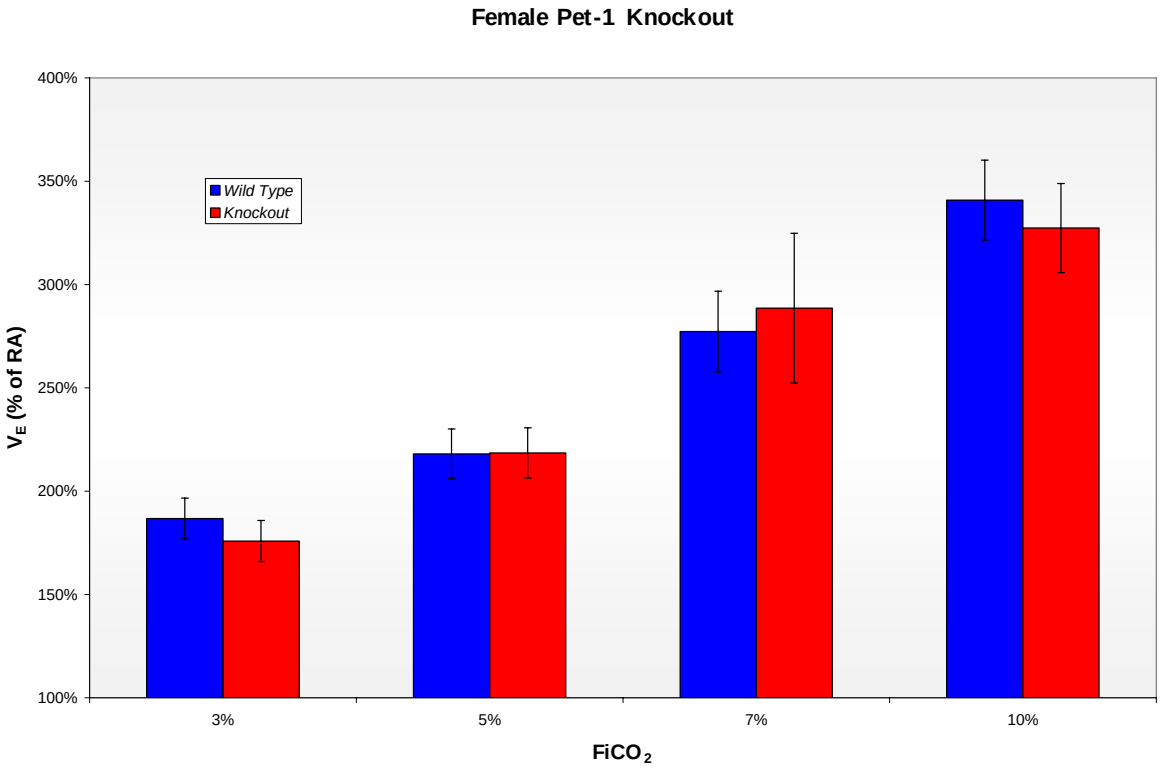

Figure 1 (c)

Sex Hormones and Respiration

Very early evidence for the effect of sex hormones on respiration was the observation that pregnant women have decreased alveolar and arterial P_{CO2} (39, 40). The same effect was also seen during the luteal of the menstrual cycle (41) and these cyclic fluctuations disappeared after menopause (42). These observations were then correlated with progesterone (43), and further in pregnant women as the log of serum progesterone (44).

Progesterone was then shown to be a respiratory stimulant in normal, healthy men (45) in multiple physiologic states including exercise and sleep (46). Clearly men do not have the progesterone influence of pregnancy, but the one instance of a hyper progesterone and estrogen state in males is cirrhosis of the liver. Cirrhotics have long been known to have a baseline respiratory alkalosis, and when a group of patients with liver disease were divided into those with severe or mild hyperventilation those with greater hyperventilation had higher serum levels of estrogen and progesterone (47). It is interesting to note that the levels of progesterone that apparently stimulated breathing in cirrhotics were two orders of magnitude less than that in pregnant woman, which may reflect different sensitivities to the effect of sex hormones.

These hormones may have an effect on common respiratory diseases in humans, specifically Obstructive Sleep Apnea (OSA), which has a strong male predominance (48). Women are protected until menopause (49) - 11% of premenopausal women suffered from nocturnal desaturation, hypopneas or apneas during sleep in one study, while 60% of postmenopausal women had the same symptoms (50). There is also evidence that women taking hormone replacement therapies were protected (51).

Synthetic progestins (most commonly Medroxyprogesterone [MPA]) were therefore used as a treatment for OSA, and some studies showed improvements in blood gas levels, sleepiness, and apneic events in males (52-54). Since estrogen increases progesterone receptors (55) in females a combination hormone replacement therapy incorporating estrogen reduced sleep associated breathing disorders (56-58).

The ventilatory effects of estrogen and progesterone have also been duplicated in other animal systems. Guinea pigs (59), Jersey cows (60), rabbits (61), and goats (62) hyperventilate when they are pregnant. However, chronic progesterone to male rabbits and goats did not increase ventilation (63). Female rats do not have a ventilatory response to MPA alone, although when MPA is combined with estrogen the number of progesterone receptors increases and ventilation increases (64). This finding was duplicated in male rats, where only the combination of estrogen and a progesterone analog had an effect on arterial blood gas parameters (65). Work in cats showed the same requirement for estrogen and progesterone and further refined the model by blocking the response with an estrogen receptor antagonist, progesterone receptor antagonist, and inhibitors of protein and RNA synthesis (66). Therefore it was concluded that the ventilatory response is dependent upon both receptors as well as gene expression, consistent with the known mechanism of steroid receptor activation.

There are a few mechanisms possible for the ventilatory changes seen as a result of female sex hormones. The most attractive for the purposes of the current study is that estrogen and progesterone alter the hypercapnic ventilatory response (HCVR) ie) they influence central chemoreception. In men, administration of progesterone stimulated ventilation and also increased the HCVR versus placebo without changing the hypoxic

ventilatory response (45). In women, the change in ventilation in the luteal phase of the cycle was shown to be due to a difference in the carbon dioxide sensitivity (67). In post-menopausal women administration of estrogen and progesterone combined with mild exercise (to increase metabolic rate) caused an increase in ventilation that mimicked the effects of pregnancy. The authors showed that both progesterone alone and the combination of estrogen and progesterone caused a significant change in the HCVR (68). Finally, in pregnant women themselves, a challenge with hyperoxic hypercapnia showed a significant difference in both the HCVR and the threshold at which this response was activated (69).

Certainly other mechanisms besides a change in the HCVR could be involved. Both male and female rats express ERα, ERβ, and androgen receptors in the hypoglossal and phrenic motoneurons and nuclei, although no progesterone binding was seen (70). Sex hormones could be functioning as amplifiers of the normal HCVR simply by driving stronger motor output. They could also change the basic respiratory rhythm, however, there is no evidence that respiratory pattern generation generated by the pre-Bötzinger complex is altered by sex hormones (38).

Female sex hormones clearly have an influence on respiratory parameters across a wide variety of species, and perhaps even a specific effect on the hypercapnic ventilatory response. Therefore we hypothesized that supplementation of the male Pet-1 knock-out mice with physiologic levels of estrogen and progesterone would partially or completely restore the deficiency in their normoxic hypercapnic ventilatory response.

Statement of Purpose

The specific aims of this study were therefore:

1) To confirm the deficient ventilatory response of male Pet-1 knockout mice in response to normoxic hypercapnic challenges through whole-body plethysmography
2) Supplement the same male Pet-1 knockout mice with estrogen and progesterone and quantify the change, if any, in their hypercapnic ventilatory response as a result of hormone treatment

in order to:

Prove or disprove the hypothesis that the female sex hormones estrogen and progesterone are responsible for the sex differences in the hypercapnic ventilatory response of male and female Pet-1 knockout mice.

Methods

The protocol followed for these experiments was based upon a modified experimental design for previously published data (36) and approved for use by the Yale Animal Care and Use Committee.

10 male PET-1 mice (5 PET-1 -/-; 5 PET-1 +/+) generated from a breeder colony generously donated by the Deneris lab at Case Western were utilized for these experiments. All animals were kept in the Yale Animal Care Facilities and provided standard care – 12 hour diurnal cycles with freely available food and water.

Plethysmography

All plethysmography experiments were carried out in the following manner with the investigator blind to the mouse genotype. The mouse to be studied was weighed prior to experimentation and the ambient barometric pressure recorded. The mouse was then placed inside the airtight plethysmography chamber [Figure 2 (a)] which was connected to a gas manifold, a vacuum with a flow regulator, and a pressure transducer. When sealed, the plethysmography chamber and pressure transducer records a change in pressure that varies directly with the animal's breathing as the inhaled gas is warmed and humidified in the lungs (71). A custom designed Matlab 7.0.2 program then converted the output voltage of the transducer into a linear trace of the pressure in the chamber, while simultaneously recording the voltage at a sampling rate of 100 Hz. The pressure in the box could therefore be monitored to insure an accurate ventilatory signal and prevent a buildup of negative or positive pressure in the closed system. An extra chamber with a

needle valve connected to the main plethysmography chamber provided an additional pressure release point while buffering the chamber against sudden changes in atmospheric pressure (such as a door opening in the laboratory). This design is consistent with standard procedures for plethysmography experiments.

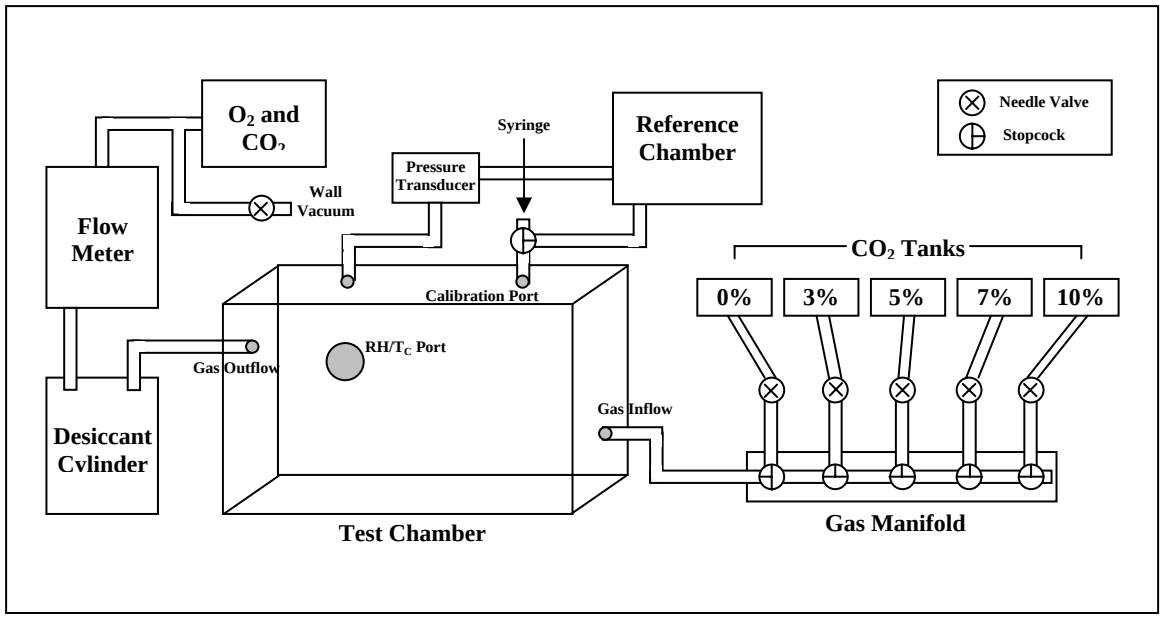

Figure 2 (a)

When placed in the plethysmography chamber each mouse was then exposed to a set protocol of gases [Figure 2 (b)]. Each exposure was set at ten minutes to allow the animal's breathing to stabilize while minimizing fatigue. Ten minutes at room air (21% O_2, balance Nitrogen) was followed by ten minute exposures to CO_2 mixes containing 3%, 5%, 7%, and 10% CO_2 respectively with 21% O_2 and balance Nitrogen. Every CO_2 exposure was alternated with ten minutes of room air exposure to allow for multiple sampling of the baseline ventilation and prevent respiratory fatigue secondary to prolonged hypercapnic exposure. Connected in series with the plethysmography

chamber was an O_2 and CO_2 analyzer which could verify the achievement of a stable CO_2 exposure level. The mouse was continually monitored throughout the experiment to maintain a state of quiet wakefulness, as sleep has an effect on the ventilatory response to hypercapnia (72, 73). Ten second time periods of quiet breathing were noted throughout the experiment to ensure usable data and marked for further analysis. At the end of each set of plethysmography experiment a 1cc syringe was used to repeatedly inject 0.1cc of air into the chamber with and without the mouse. This established a known volume to voltage relationship which could then be used to calculate absolute tidal volumes for each mouse.

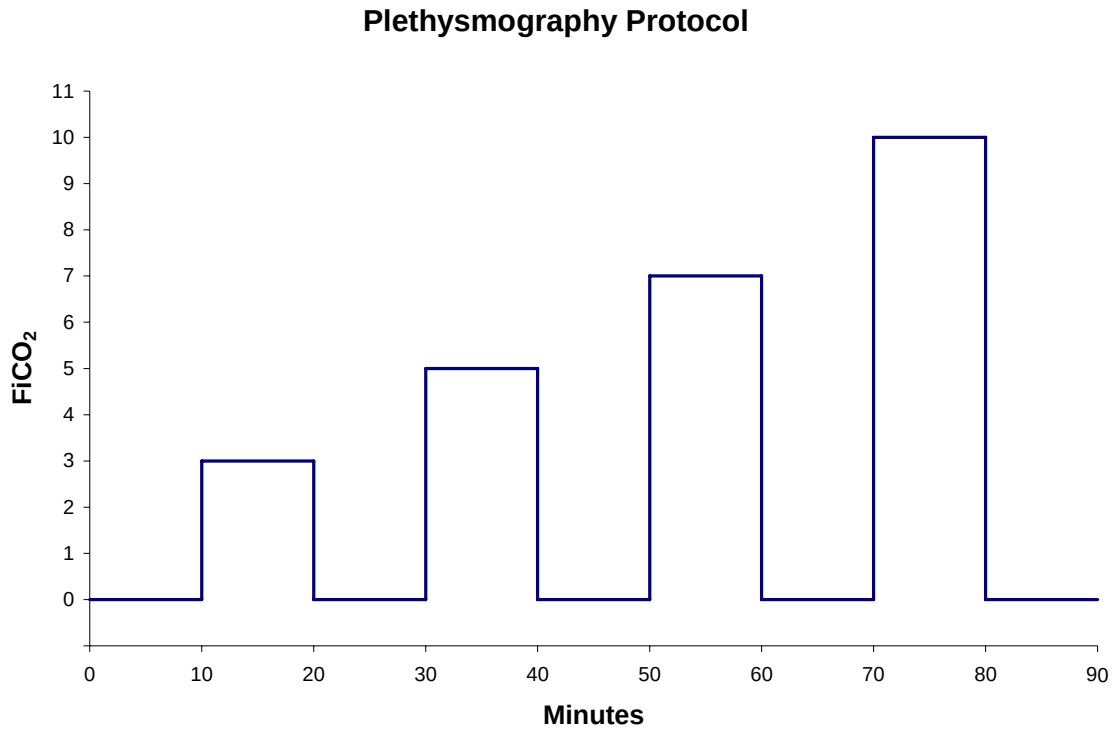

Figure 2 (b)

Hormone Exposure

In order to test the hypothesis that the deficiency seen in the ventilatory response of the male mice was a result of the relative lack of female sex hormones, the male mice were supplemented with estrogen and progesterone in the following manner. Each mouse served as its own control for these experiments, and was therefore studied in the plethysmography chamber as outlined above on Day 0, immediately prior to commencing hormone supplementation. The goal of hormone supplementation was to recapitulate as closely as possible the normal serum levels of both female sex hormones in the male mice, rather than administer a supra-physiologic dose. Normal female mice in their normal estrus cycle have a serum estrogen between 10 and 30 pg/ml (74), and serum progesterone between 10-60 ng/ml (75).

The adult male mice were treated with estrogen through their drinking water, as oral estrogen has good bioavailability. 17β-estradiol was dissolved at a 20 mM concentration in 95% ethyl alcohol (EtOH) and then diluted to a concentration of 2,500 nM in the standard chlorinated drinking water of the mice. Their daily water intake was then substituted with the estrogen drinking water. Preliminary supplementation in non-experimental mice had already been undertaken to show that Pet-1 mice drank the supplemented water and achieved their daily intake needs without dehydration. Estrogen drinking water at 2,500 nM dose has been shown to correspond to an estrogen serum level of 38 pg/ml – at the high range of estrogen throughout the normal mouse estrus cycle (76). Each experimental mouse had access to the estrogen drinking water for 48 hours, immediately following their control protocol in the plethysmography chamber.

Stable plasma levels of estrogen would be expected within six hours of oral administration, well within the exposure duration for this experiment (77).

As progesterone is extensively metabolized by the liver on first pass and therefore has poor oral bioavailablity it is most commonly administered by intramuscular injection or subcutaneous capsules. Although progesterone has a half-life in circulation of only 5 minutes, suspension in sterile sesame oil allows for a depo-like effect with slowly declining serum levels after a single injection or implantation (78, 79). Experiments in humans have shown that injection at a dose of 1.5 mg/kg achieves a serum level between 5 and 50 ng/ml over 48 hours (78), slightly lower than the normal level in mice. The male mice therefore received a subcutaneous injection of 2 mg/kg of progesterone suspended in 0.1 mL of sterile sesame oil one hour before the plethysmography experiment.

It was noted during the course of these experiments that following the injection some sesame oil would be expressed back out of the injection site, usually following vigorous motor activity. While this would have reduced the total progesterone dose received by the mouse, the pharmacokinetics of progesterone is such that the dose injected has less of an impact on peak plasma levels than on the duration of the elevation. In humans, a dose one quarter of the dose utilized in these experiments achieves similar peak plasma levels of progesterone, however, the duration of the elevation is shortened from 24 hours to 12 hours (78). Qualitative assessment after depositing 0.1 mL of sesame oil directly onto the skin of a mouse suggested that much greater than 50% of the injected dose remained subcutaneous throughout the experiment. As the experiments

lasted for approximately 3 hours post-injection, even 50% of the injected dose would produce target plasma levels for many hours longer than required.

Data Analysis

The voltage vs. time data was recorded for each experiment in MatLab file format, according to the previously described protocol. Another custom-designed MatLab 7.0.2 was used to access this file and display it in plotted form, where the data could be explored for ten second segments of quiet breathing, without mouse movement, sniffing, or sighs. Breaths during the segment were identified by the peaks and troughs of the voltage, and the interspike interval and amplitude was averaged across all breaths in the segment. This procedure was repeated across all CO_2 exposure levels with the goal of finding at least six ten second segments of useable data during each gas exposure. The mean interbreath interval and amplitude along with standard deviations were then calculated for each gas exposure by averaging the values from the ten second segments. The final room air interbreath interval and amplitude was obtained by an average of all five ten minute exposures to minimize the effect of escalating CO_2 on baseline ventilation. The volume calibration performed with the mouse in the chamber was then used to assist calculations of the absolute tidal volumes from the average voltage deflection per breath in the following manner.

Since the pressure exerted by any gas varies with its temperature and partial pressure of H_2O, the air warmed and humidified by the lungs expands, and increases the pressure in the sealed chamber upon inhalation. The chamber temperature (Tc) and relative humidity (both measured parameters) are used to calculate the partial pressure of

water in the chamber (Pc_{H2O}). The animal's pulmonary temperature is assumed to be equal to the animal's body temperature (Ta), with the relative humidity of the inspired air equal to the saturation humidity at that temperature. Because the animal's temperature was not directly measured in these experiments, it was assumed to be 37° C for all animals and the water pressure of pulmonary air calculated (Pa_{H2O}). The known calibration volume (Vcal) injected into the chamber that causes a known pressure change (Pcal) was used to scale the following formula derived from the ideal gas law, where Pb is the ambient barometric pressure and where A equals the voltage deflection of the animal's breath (80):

$$V_T = A\ (Vcal / Pcal)\ [Ta(Pb - Pc_{H2O})] /\ [[Ta(Pb - Pc_{H2O})] -\ [Tc(Pb - Pa_{H2O})]]$$

As the frequency of respiration is easily generated from the interbreath interval (Frequency = 1 / IB Interval), absolute ventilation (V_E) can be calculated by taking the frequency multiplied by tidal volume.

$$V_E\ (mL / minute) = Frequency * V_T$$

Since the absolute tidal volume (mL) depends on the size of the mouse and the relative size of its lungs, pooling data from all mice require that both V_T and V_E be scaled. A surrogate and more easily measured variable for total lung capacity is the weight of the mouse. V_T and V_E are therefore reported as adjusted for mass - V_T (mL / g) and V_E (mL / minute/ g).

For every mouse a respiratory frequency, V_T, and V_E were calculated for each ten minute gas exposure (Room Air, 3%, 5%, 7%, and 10% CO_2). As explained above, in order to minimize variability in the control measurement as a result of escalating CO_2, the final control frequency, V_T, and V_E were obtained by an average of all five control exposures. These results were then pooled and sorted by genotype with statistical significance determined by the Paired Student's T-test.

Results

Consistent with the previous experiments on Pet-1 mice, a deficiency in their ventilatory response to normoxic hypercapnia was seen across multiple CO_2 levels. Seen side-by-side, the ventilatory tracing of a Pet-1 knockout mouse can be readily distinguished from a wild-type mouse across multiple CO_2 exposures [Figure 3].

Before hormone treatment each genotype's ventilatory response was characterized according to tidal volume, frequency, and ventilation (V_E) [Figure 4 (a), (b), (c)]. Little difference was seen in tidal volume between the wildtype versus knockout – at Room Air (1.54 ± 0.08 vs. 1.37 ± 0.11 ml/g), 3% CO_2 (2.00 ± 0.18 vs. 1.72 ± 0.13 ml/g), 5% CO_2 (2.18 ± 0.16 vs. 1.83 ± 0.17 ml/g), 7% CO_2 (2.31 ± 0.16 vs. 2.13 ± 0.10 ml/g), and 10% CO_2 (2.48 ± 0.17 vs. 2.49 ± 0.12 ml/g) these differences were not statistically significant ($p > 0.05$ for all data points).

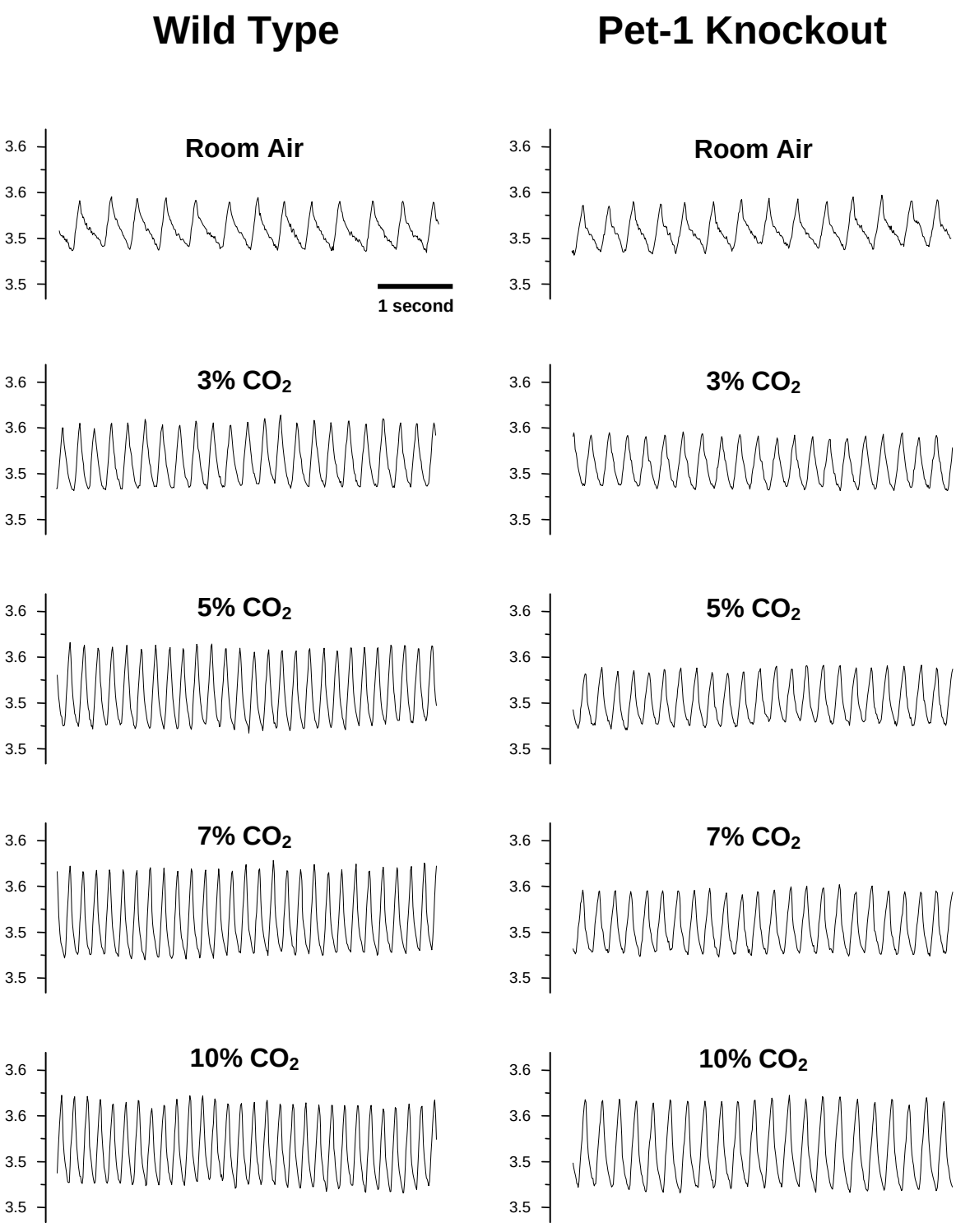
Wild Type
Pet-1 Knockout
Room Air
3% CO2
5% CO2
7% CO2
10% CO2
3.6
3.5
1 second

Figure 3

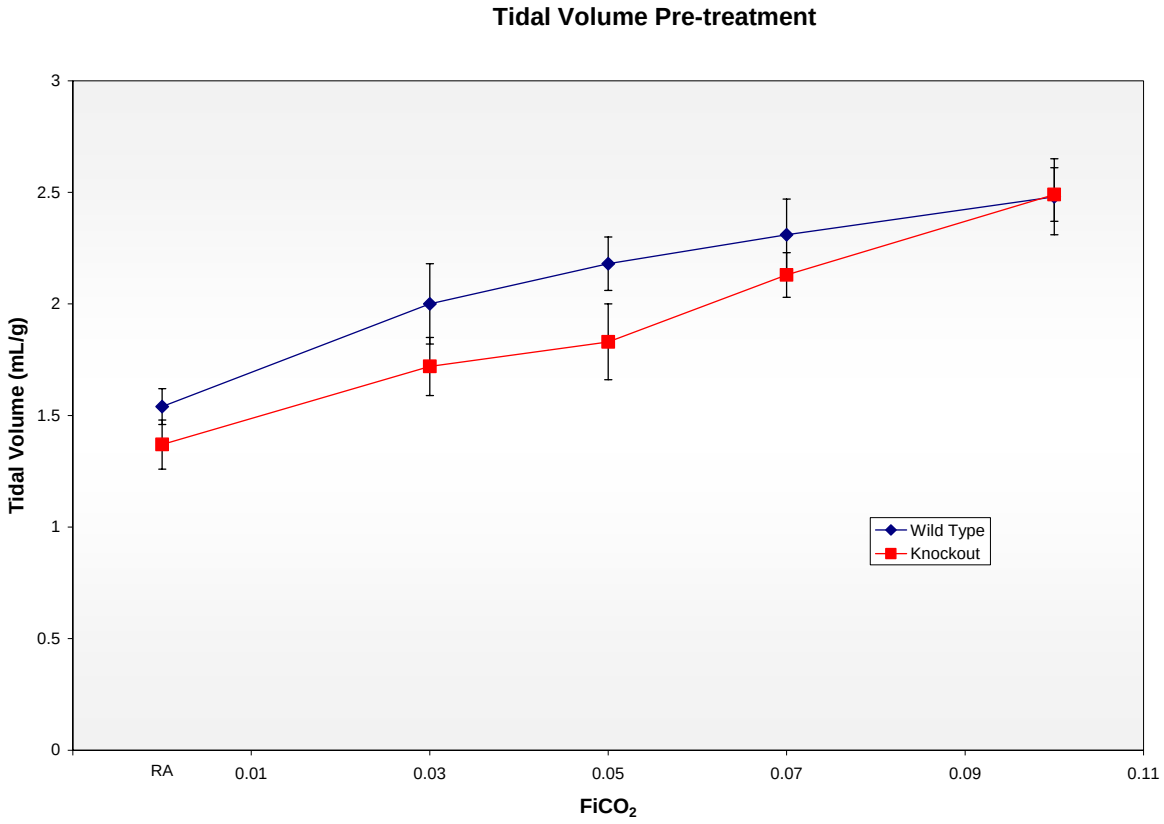

Figure 4 (a)

A different result was seen for frequency. Across most of the range of CO_2 exposure there was a statistically significant difference between the wildtype and knockout mice. At 3% CO_2 (282 ± 10 vs. 242 ± 8 breaths/minute; p <0.05), 5% (301 ± 14 vs. 260 ± 8 breaths/minute; p <0.05), and 7% (315 ± 14 vs. 270 ± 11 breaths/minute; p = <0.05) the difference in frequency was statistically significant. As would be expected if the baseline ventilation was unaffected, the control respiratory frequency was not statistically significant between the two groups (179 ± 10 vs. 167 ± 10 breaths/minute; p = NS). Interestingly, the breathing frequency was similar between the two groups at 10% CO_2 as well (308 ± 12 vs. 290 ± 7 breaths/minute; NS).

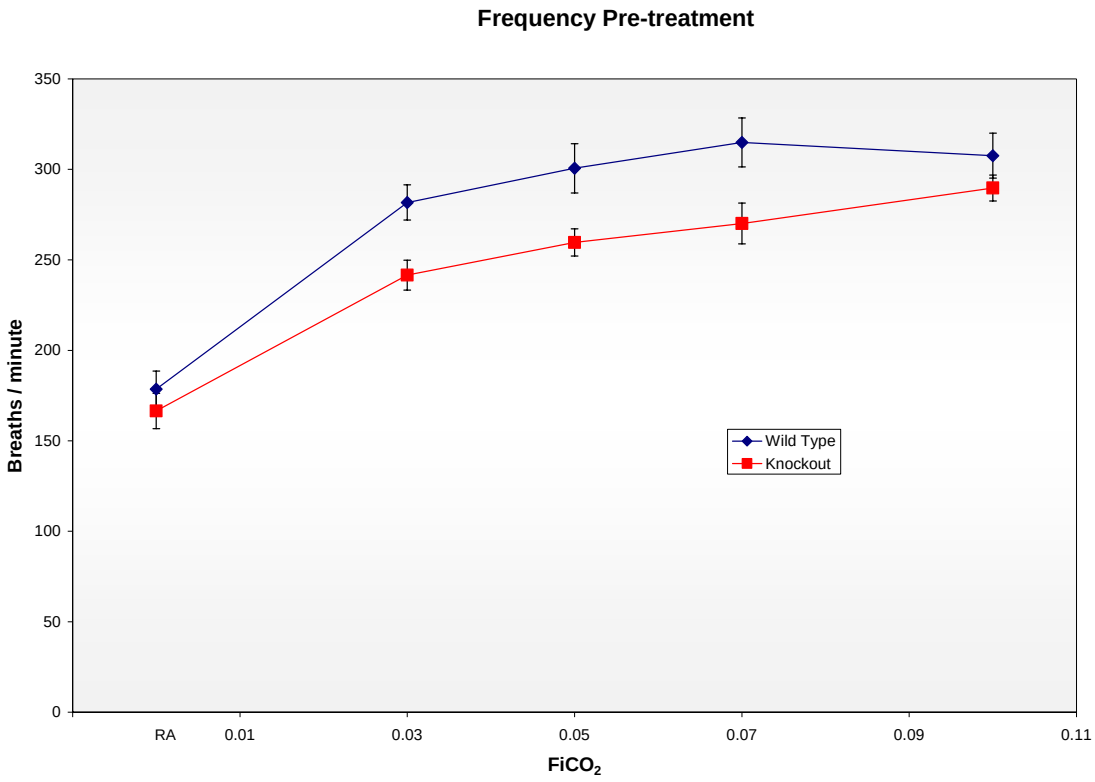

Figure 4 (b)

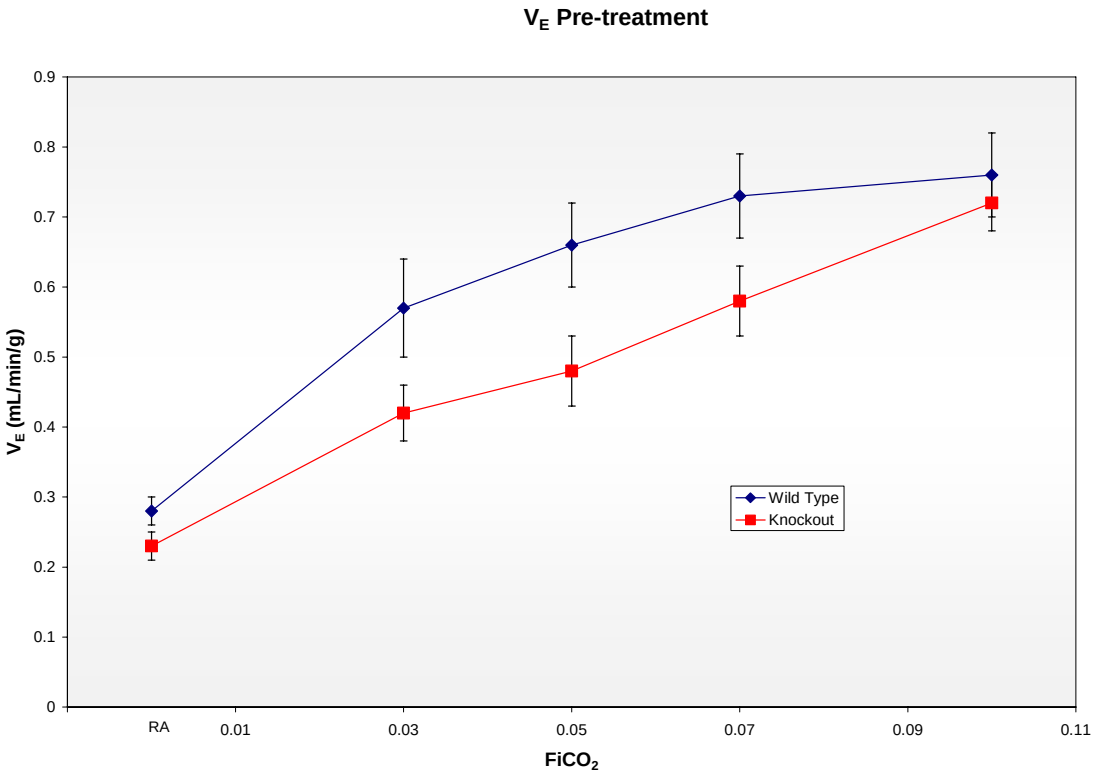

Figure 4 (c)

The difference in respiratory frequency therefore accounts for most of the difference in overall ventilation between the two groups. There was a trend towards lower overall ventilation under control conditions in the knockouts (028 ± 0.02 vs. 0.23 ± 0.02 ml/min/g; NS). The same trend was noted across all gas exposures up to 10% CO_2 although it achieved statistical significance only at 5% CO_2: 3% CO_2 (0.57 ± 0.07 vs. 0.42 ± 0.04 ml/min/g; NS), 5% CO_2 (0.66 ± 0.06 vs. 0.48 ± 0.04 ml/min/g; $p < 0.05$), and 7% CO_2 (0.73 ± 0.06 vs. 0.58 ± 0.05 ml/min/g; NS). At 10% CO_2, the knockout's ventilatory response has clearly recovered to equal that of the wildtype (0.76 ± 0.06 vs. 0.72 ± 0.04 ml/min/g; NS). This result could suggest that mechanisms other than the serotonin system could assist in the ventilatory response under extreme duress and will be more fully discussed later.

Graphing the results as percent change from the control exposure gives a different perspective on these results [Figure 4 (d)]. The hypercapnic ventilatory response (HCVR) is usually determined from the slope of the CO_2 response across multiple CO_2 levels ($\Delta V_E/\Delta F_ICO_2$). As seen from the % Response curve for the wildtype and knockout mice each genotype has a change in ventilation from their baseline, although as discussed previously, the absolute ventilation across multiple carbon dioxide levels trends lower at all hypercapnic exposures other than 10% CO_2.

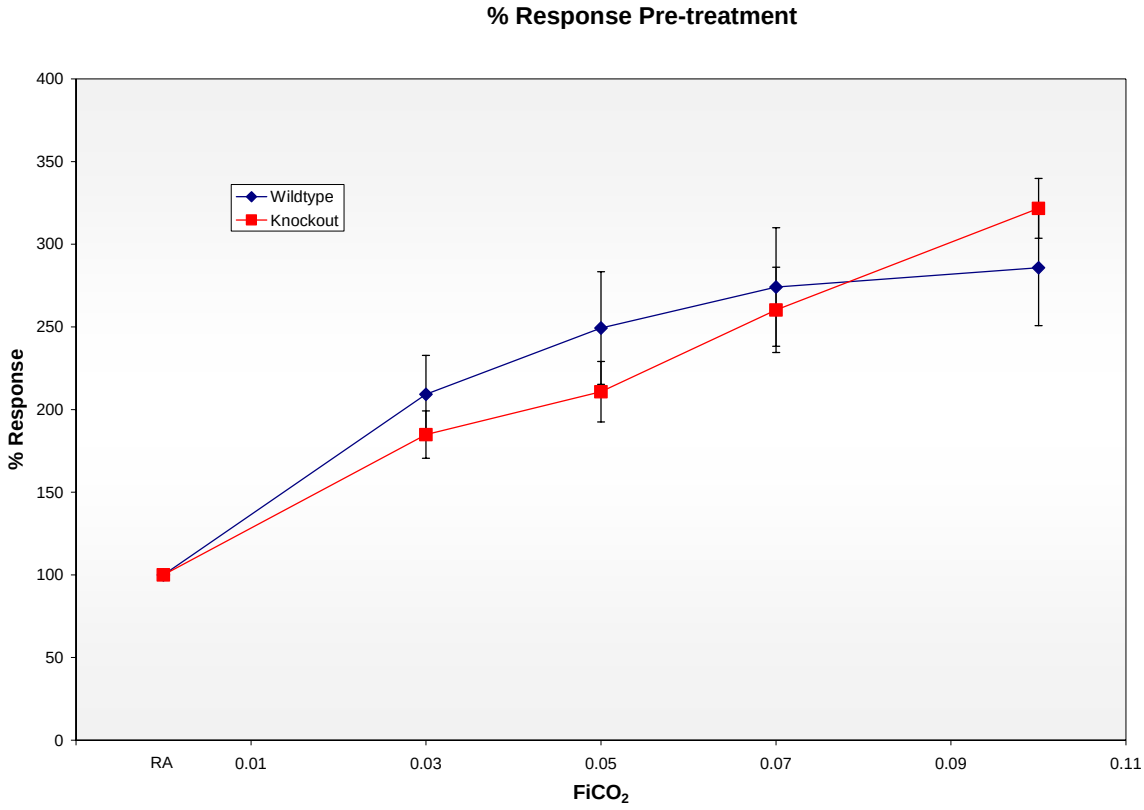

Figure 4 (d)

Hormone Therapy

After acute treatment with hormone therapy the same ventilatory parameters were measured again [Figure 5 (a), (b), (c)].

As seen before hormone therapy, there was no difference between the wild type and knockout groups in tidal volume, nor were the tidal volumes increased consistently as a result of the hormones. At all exposures there was not a statistical difference between the groups: control (1.48 ± 0.05 vs. 1.35 ± 0.07 ml/g; NS), 3% CO_2 (1.88 ± 0.07 vs. 1.62 ± 0.09 ml/g; NS), 5% CO_2 (1.95 ± 0.09 vs. 1.95 ± 0.1 ml/g), 7% CO_2 (2.07 ± 0.12 vs. 2.20 ± 0.12 ml/g), and 10% CO_2 (2.29 ± 0.1 vs. 2.50 ± 0.15 ml/g).

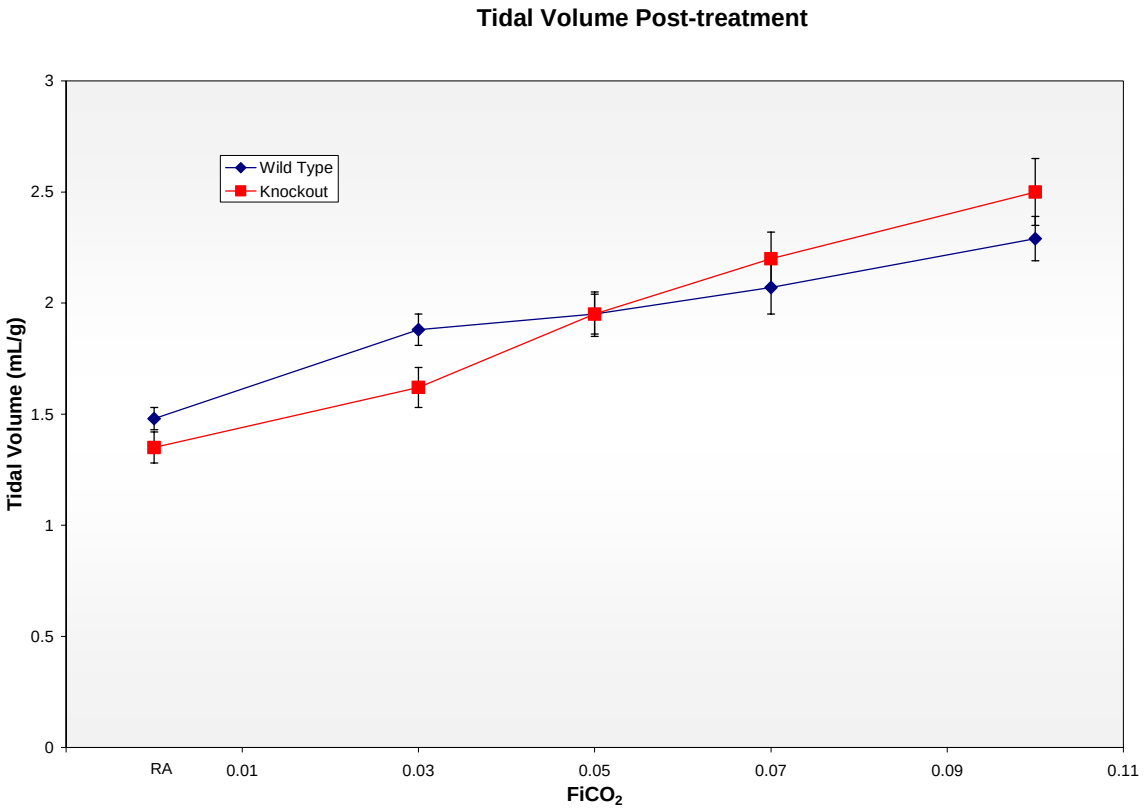

Figure 5 (a)

Similar to the results seen before hormone therapy, the ventilatory frequency was the respiratory parameter that showed the most difference between the two groups. At room air (195 ± 8 vs. 154 ± 11 breaths/minute; $p < 0.05$) and all carbon dioxide exposures up to 7% CO_2 there was a significant difference between the two genotypes: 3% CO2 (294 ± 7 vs. 225 ± 10 breaths/minute; $p < 0.001$), 5% CO2 (300 ± 10 vs. 248 ± 6 breaths/minute; $p < 0.005$). At 7% CO2 (308 ± 11 vs. 282 ± 13 breaths/minute; NS) and 10% CO2 (307 ± 9 vs. 278 ± 13 breaths/minute; NS) there was no statistical significance between the groups. Therefore, hormone therapy with estrogen and progesterone did not alter the basic characteristics of the differences in hypercapnic ventilatory response between the two genotypes.

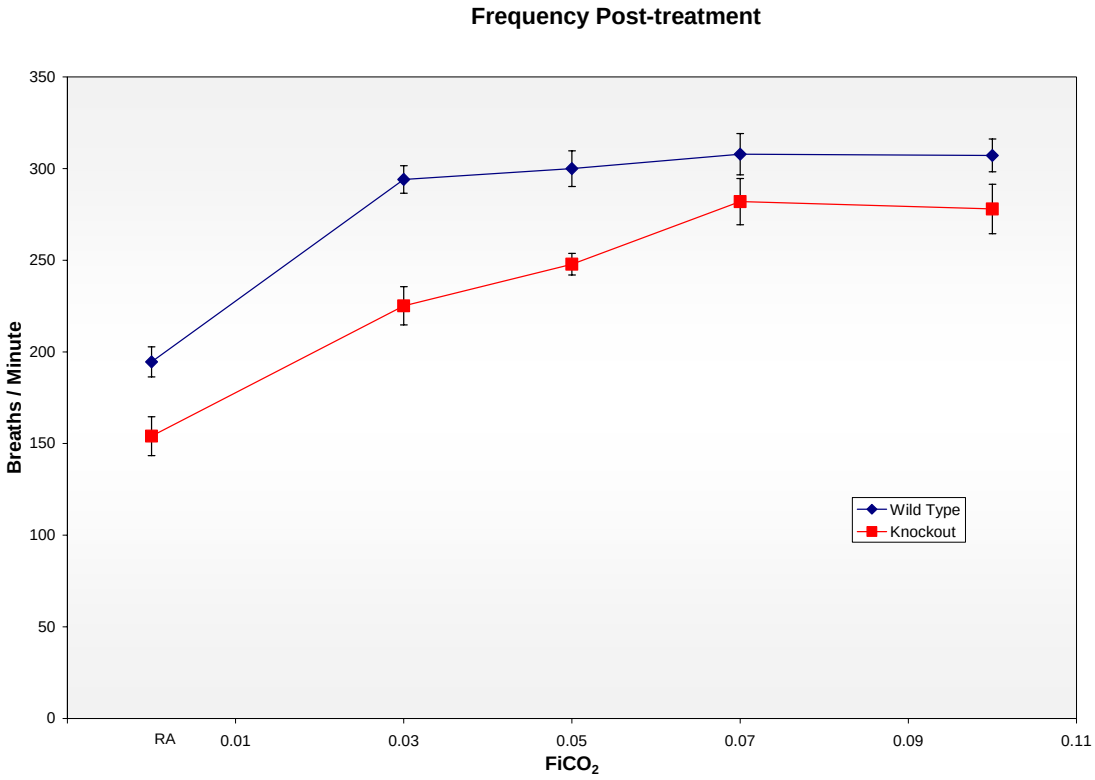

Figure 5 (b)

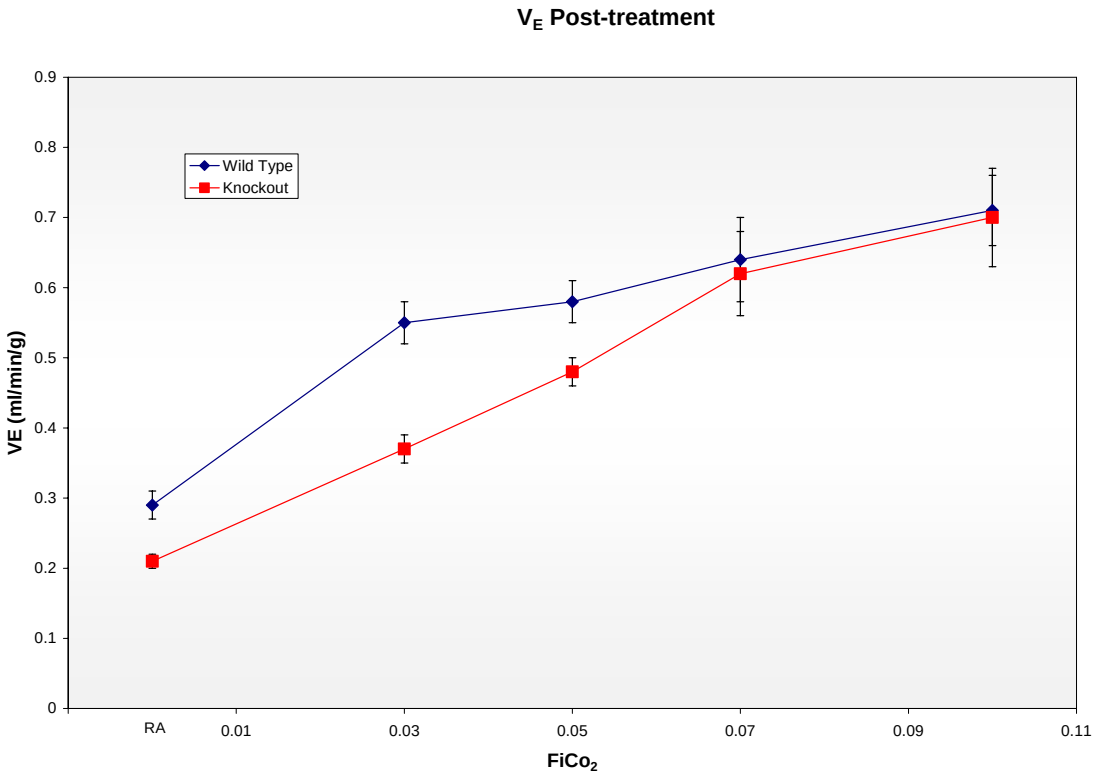

Figure 5 (c)

Total ventilation continued to be reduced in the knockout mice at control and lower CO_2 levels even after treatment with estrogen and progesterone, although the ventilation recovery seen in the knockout mice at the highest CO_2 exposure was now obvious at both 7% and 10% CO_2. The difference between the groups was statistically different at room air (0.29 ± 0.02 vs. 0.21 ± 0.01 ml/min/g; $p < 0.005$), 3% CO_2 (0.55 ± 0.03 vs. 0.37 ± 0.02 ml/min/g; $p < 0.0005$), and 5% CO_2 (0.58 ± 0.03 vs. 0.48 ± 0.02 ml/min/g; $p < 0.05$). After treatment total ventilation at both 7% CO_2 (0.64 ± 0.06 vs. 0.62 ± 0.06; NS) and 10% CO_2 (0.71 ± 0.05 vs. 0.70 ± 0.07 ml/min/g; NS) was not statistically different. This result could suggest that the mechanism that responds to higher levels of hypercapnic stress is hormone sensitive.

This finding is emphasized by a plot of the ventilatory response compared as a percent to the baseline room air breathing [Figure 5 (d)]. Before treatment the knockout group had a similar slope to the wildtype, but under the influence of female hormones it shows a more robust response, especially at the higher CO_2 levels. However, this trend was not statistically significant: 7% CO_2 (227 ± 27 vs. 305 ± 33 % response; NS) and 10% CO_2 (250 ± 25 vs. 344 ± 41 % response; NS).

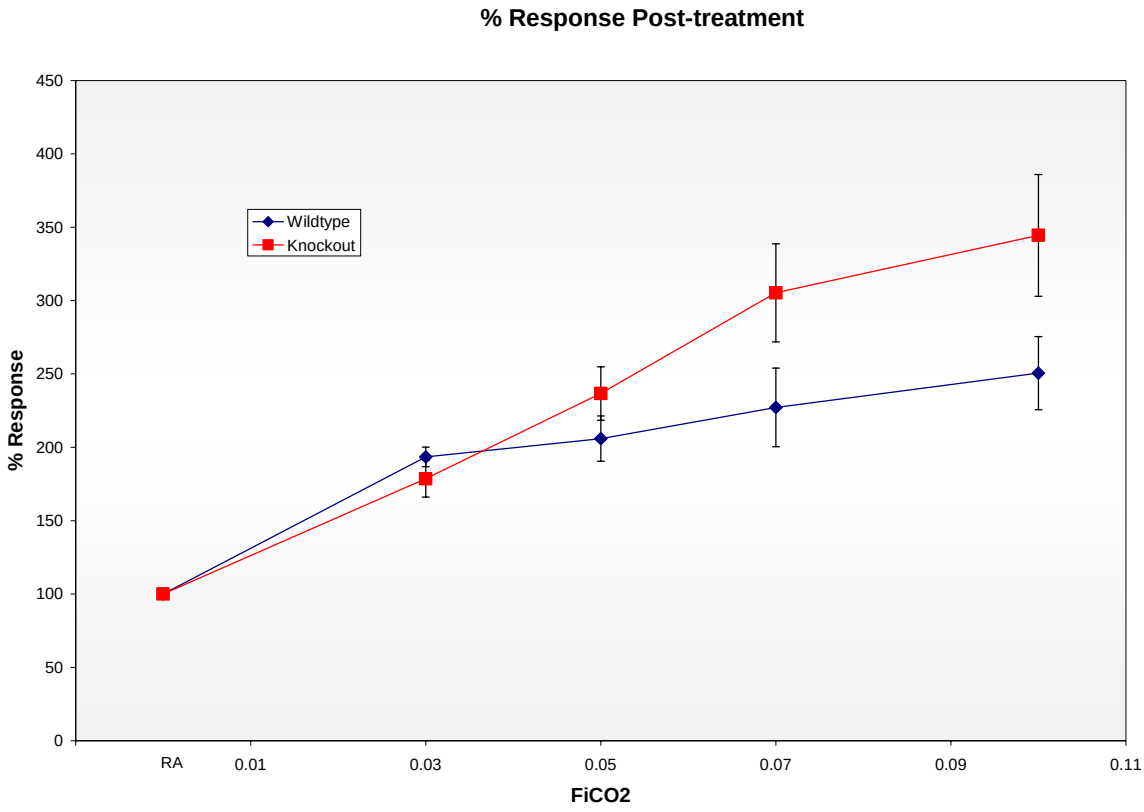

Figure 5 (d)

When comparing the absolute ventilation with and without hormones there are little differences between the wildtype or knockout groups to suggest that absolute ventilation changed as a result of hormone treatment [Figure 5 (e)]. At room air and across all CO_2 exposures there was no significant change in V_E in the wildtype group: room air (0.28 ± 0.02 vs. 0.29 ± 0.02 ml/min/g), 3% CO_2 (0.57 ± 0.07 vs. 0.55 ± 0.03 ml/min/g), 5% CO_2 (0.66 ± 0.06 vs. 0.58 ± 0.03 ml/min/g), 7% CO_2 (0.73 ± 0.06 vs. 0.64 ± 0.06 ml/min/g), and 10% CO_2 (0.76 ± 0.06 vs. 0.71 ± 0.05 ml/min/g). Therefore it does not appear that this level of acute supplementation with female hormones stimulated breathing in the wildtype mice.

Similarly there was no difference in absolute ventilation in the knockout group as a result of hormone treatment when directly compared to the pretreatment ventilation: room air (0.23 ± 0.02 vs. 0.21 ± 0.01 ml/min/g), 3% CO_2 (0.42 ± 0.04 vs. 0.37 ± 0.02 ml/min/g), 5% CO_2 (0.48 ± 0.05 vs. 0.48 ± 0.02 ml/min/g), 7% CO_2 (0.58 ± 0.05 vs. 0.62 ± 0.06 ml/min/g), and 10% CO_2 (0.72 ± 0.04 vs. 0.70 ± 0.07 ml/min/g).

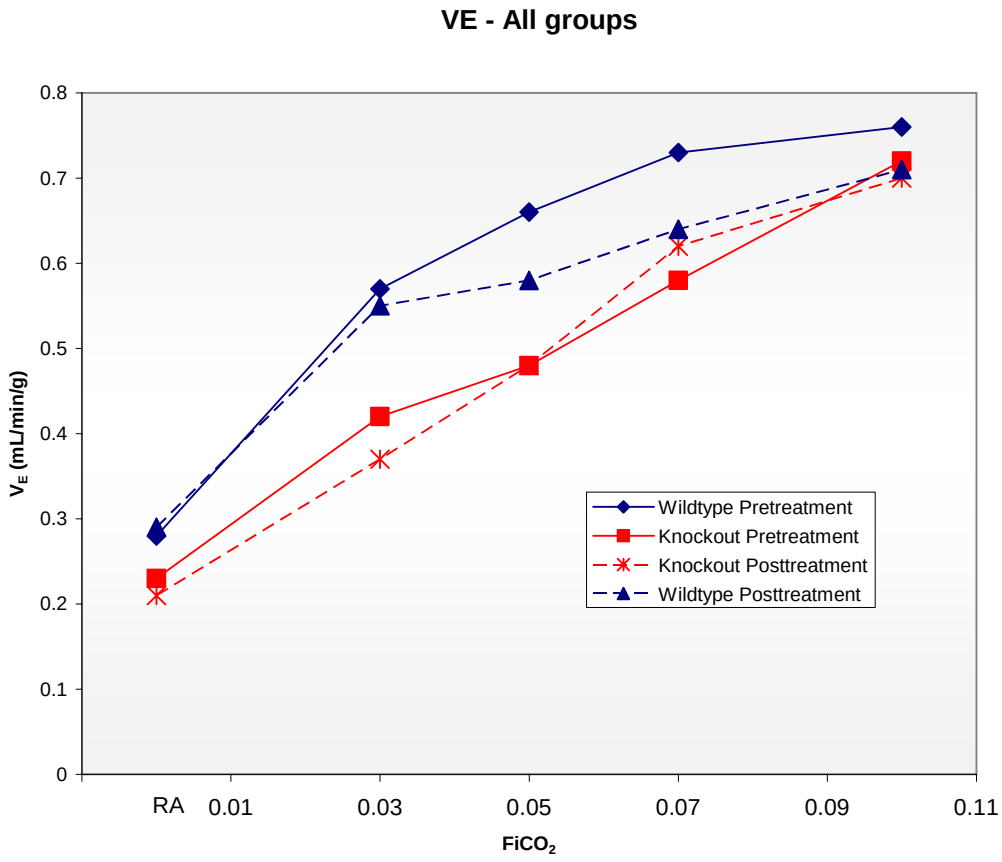

Figure 5 (e)

The higher percentile response seen after hormone treatment in the knockout group is due to a combination of factors – a slight decrease in the room air ventilation for the knockout group versus a slight increase the wildtype, along with a small *decrease* in the overall response of the wildtype that magnifies the effect of an identical response in

the knockout group. Therefore it can be concluded that supplementation with female sex hormones did not significantly impact the absolute ventilation of either the wildtype or knockout Pet-1 mice, but there is evidence that it changed the relative response between the groups at higher CO_2 exposures.

Discussion

The goals of this study were to 1) confirm and further define the deficiencies of the Pet-1 male's response to normoxic hypercapnia and to 2) determine if this deficiency could be accounted for and corrected by physiologic supplementation with female sex hormones.

In a confirmation of previously obtained results, the Pet-1 knockout mice showed a statistically significant deficiency in their total ventilation, mainly at lower hypercapnic exposures. The trend seen pre-treatment did not achieve statistical significance except at 5% CO_2 most likely because of the small sample size. However, as their room air ventilation was also depressed relative to the wildtype mice, their ventilatory response seen as a percentage of baseline was not different than the respiratory response of the wildtype. This raises the important question of the most accurate determinant of the hypercapnic ventilatory response. The HCVR is most commonly expressed as the slope of V_E, which takes into account both the baseline ventilation and the hypercapnic ventilation; by this measure the Pet-1 knockout mice do not show a difference in their HCVR.

Despite this result, there is a clear phenotype for the male Pet-1 knockout mice compared to their wildtype kin. Their total ventilation is reduced both at room air and for all hypercapnic exposures except 10% CO_2. As is readily evident from the V_E graph, the decrease from the wildtype ventilation is not equivalent across all exposures, as might be expected if the loss of serotonergic neurons interfered with the fundamental mechanisms of breathing such as respiratory pattern generation, phrenic discharge, or diaphragmatic function. At room air, the knockout's V_E is 82% that of wildtype, 74%, 73%, and 79% at 3%, 5% and 7% CO_2 respectively and finally 95% at 10% CO_2. These variable results argue strongly that the disruption of the serotonin system in the Pet-1 mice does have an effect on the hypercapnic ventilatory response, but also that this effect is not uniform across the possible range of hypercapnic stressors. It could be concluded therefore, that the serotonin system plays an important role in maintaining baseline ventilation and the response to CO_2 at lower exposure levels, and that a secondary hypercapnic response independent of the serotonin system can assist in driving ventilation at higher carbon dioxide stresses.

There is a precedent for this result in other knockout genotypes also deficient in the serotonin system. Mice deficient in Lmx1b (a transcription factor necessary for neuronal development (81) driven by the Pet-1 promoter are more severely deficient in serotonin neurons than the Pet-1 knockout mice. These mice lack up to 99% of their serotonergic neurons in all serotonin nuclei. When tested in whole body plethysmography in a fashion similar to the Pet-1 mice, although using hyperoxic hypercapnia to disable peripheral chemoreceptors rather than normoxic hypercapnia, these mice show a more profound deficiency in their HCVR across a range of CO_2

similar to the results reported here [Figure 6]. The slope of V_E is linear (representing the HCVR) until 10% CO_2, where a more robust ventilatory response is seen. This biphasic response suggests that the serotonin system is functioning as a central chemoreceptor responsible for driving a ventilatory response to CO_2, but also that secondary neural systems can "rescue" the response under severe enough acidosis.

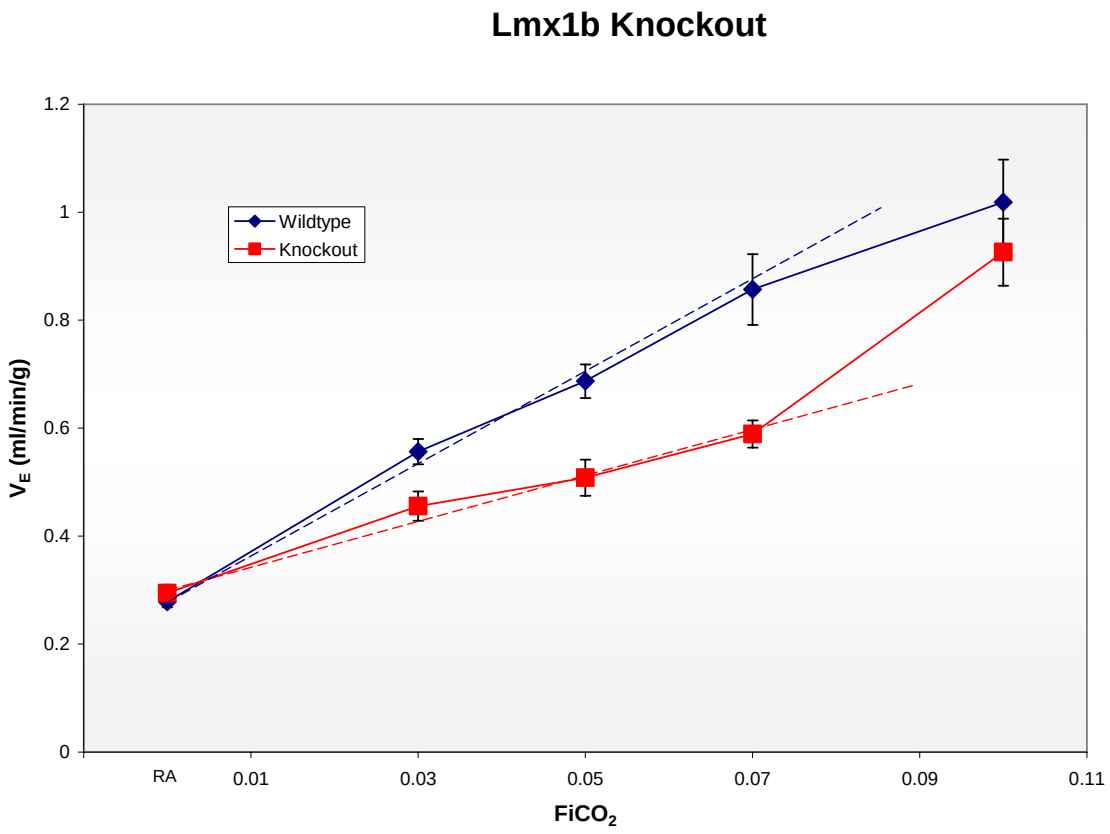

Figure 6

Seen in this context, the results from the current study support the theory of the serotonin system as a central chemoreceptor, albeit one whose effect is not constant across the entire range of hypercapnic challenges. However, the 70% loss of serotonin neurons appears to have only resulted in a ~25% loss of ventilatory response. What

could account for this difference if the hypothesis that serotonin contributes to the HCVR is accepted?

First, the experiment performed here took place under normoxic conditions. As has been well-described, peripheral chemoreceptors located in the carotid body provide the majority of the ventilatory response to hypoxia, but they also modulate the response to hypercapnia through changes in the parasympathetic or sympathetic systems and also respond directly to high CO_2 challenges (82, 83). Performing these experiments under hyperoxic conditions would decrease the influence of the peripheral chemoreceptors and might therefore unmask more of the Pet-1 knockout phenotype.

Second, there is no reason to presuppose that the phenotypic response should be linear ie) a 70% loss of serotonergic neurons would lead to a 70% reduction in the hypercapnic response. In fact, there are good reasons to suppose otherwise. 1) Although a 70% reduction in the number of serotonin neurons is quantitatively impressive, this does not address the capacity of the remaining 30%. These remaining neurons could be simply sufficient on their own to drive a ventilatory response or they could upregulate key transporters or enzymes as a compensatory response. Work with other neurotransmitter systems has shown that a surprisingly small number of remaining neurons can support essential functions. For example, in patients with unilateral Parkinson's Disease, imaging of the unaffected contralateral side shows a loss of up to 40% of the dopamine neurons (84). 2) Chemosensitivity is an extremely important homeostatic mechanism, and in the absence of a normal serotonin system it is possible that other neurotransmitter systems that normally have a minor role in chemosensitivity could be recruited for this function. Other candidate systems could include neurons of

the locus coeruleus (85-87), retrotrapeziod nucleus (RTN) (88, 89) or the nucleus tractus solitarius (NTS) (90, 91). 3) There are also inherent difficulties in interpreting the results of knockout mice. Soon after the development of homologous recombination to eliminate target genes, many knockout mice were generated that had surprisingly normal phenotypes given the thought-to-be essential nature of the target gene (92). In many cases this phenotypic normality has been attributed to gene redundancy, recruitment of alternative systems for compensation, or the superfluous nature of the target gene. This has led to the current popularity of "conditional knockouts", where the temporal control over a gene allows for normal protein function during development, thus preventing accessory or redundant systems from compensating when the protein is removed in adult life (93). While the Pet-1 mice do exhibit a clear neuropathologic phenotype, it is not clear what the effect of a reduction of serotonin neurons during fetal and early development has on normal brain function.

It should be noted that every one of these caveats argues for a normalization of phenotype, and therefore the ~25% reduction seen in ventilation and its differential relationship to hypercapnia is an extremely strong argument for the role of serotonin neurons in normal central chemosensitivity.

Sex Hormones and Chemosensitivity

The second goal of these experiments was to address the ability of female sex hormones to account for the differences seen between male and female Pet-1 knockout mice. A combination of estrogen and progesterone was used based upon work in rats

discussed earlier that showed the need for both hormones to increase ventilation in males and females.

A comparison of pre and post-hormone treatment total ventilation (V_E) provides no evidence that the baseline ventilation of either the wildtype or the Pet-1 knockout group was affected by hormone therapy. This result is surprising given the extensive evidence that progesterone is a respiratory stimulant. Unfortunately, as there is no consensus about dosing or administration of these hormones, many of the experiments that show the stimulatory effects of progesterone use supra-physiologic doses.

In the current study progesterone was dosed at 2 mg/kg in an attempt to recapitulate normal female hormonal levels. In comparison, stimulation of ventilation in female rats was achieved with MPA dosed at 8 mg/kg x 4 days (64). In order to stimulate ventilation in male rats, they were fed TZP4238 (a progesterone analog with a potency 300-400 times that of progesterone) at 4 mg/kg, even though some respiratory effects were seen at 0.4 mg/kg (65). For the purposes of the current study, proving the ability of supra-physiologic progesterone to stimulate ventilation in Pet-1 mice would have had no relevance on the experimental question. Clearly, if the female Pet-1 knockout mice are protected by circulating sex hormones these hormones can provide adequate protection within physiologic ranges.

While the baseline ventilation was not affected, the sex hormones did appear to influence the ventilatory response at higher CO_2 exposures. The basic pattern seen pre-treatment, where ventilation at Room Air and lower CO_2 exposures was diminished, was retained only until 5% CO_2 – after hormone treatment the knockout's V_E was 72%, 67%, and 83% that of wildtype at Room Air, 3%, and 5% respectively. The ventilatory

recovery was 97% and 99% at 7% and 10% CO_2, although the caveats discussed earlier still apply – this difference was due to a small size in the absolute ventilation of the knockout along with a small decrease in the V_E of the wildtype.

If this recovery is real and due to the hormone exposure, there are two possible interpretations of this result: 1) The deficiency in the HCVR seen in the knockout mice as a result of the loss of serotonin neurons can be influenced by female sex hormones, or 2) secondary chemoreceptive mechanisms are influenced by female sex hormones and therefore "kick in" at earlier hypercapnic stresses. These are by no means mutually exclusive, and teleologically the rescue at 7% CO_2 could be explained either way. If the remaining serotonergic neurons were enhanced by female sex hormones, this effect would surely be seen when the neurons were receiving maximum extrinsic stimulation ie) at the highest CO_2 exposure. Similarly, redundant chemoreceptive neurons could be recruited at lower and lower CO_2 thresholds if they were responsive to sex hormones. Neither of these options can be ruled in or out at this point, so it can therefore be cautiously concluded that there is evidence that supplementation with physiologic levels of female hormones can restore some of the ventilatory deficiencies seen as a result of the loss of serotonin neurons.

Now that there is some experimental evidence that estrogen and progesterone can manipulate the phenotype caused by reduced serotonergic neurons it is reasonable to ask if there are other lines of evidence to support this interaction. Clear connections between the serotonergic system and sex hormones could tip the explanatory balance towards serotonergic neurons and away from the unknown redundant chemoreceptors.

Due in large part to the role of the serotonin system in psychiatric disease – mainly depression and anxiety, both of which affect females more than males (94) – the intersection between the serotonin system and sex hormones is an active area of research (95).

Estrogen and progesterone act through nuclear receptors – estrogen receptors in two major isoforms ERα and ERβ (96) and progesterone isoforms PR-A and PR-B (97). In most species studied thus far including monkey, guinea pig, and mice, ERβ is the form that is expressed most highly in the dorsal raphe. In mice, both isoforms colocalize with serotonin neurons (98), although ERβ has a higher rate of colocalization in the dorsal raphe (90%) than ERα (23%) in male mice (99). Progesterone receptors in the serotonergic neurons are upregulated by ERβ activation in both mice and monkeys and downregulated by progesterone (100, 101).

What is known about the effects on the serotonin system of activating these receptors? One key measure of serotonin activity is the level of TPH, which is the rate-limiting enzyme in the production of serotonin. When activated by estrogen, serotonin neurons upregulate TPH mRNA in both guinea pigs (102) and macaques (103), and the same effect has recently been shown in mice to be mediated through ERβ (104). Whether this increase in mRNA results in increased TPH protein is not yet known in mice, but has been shown to be the case in guinea pigs and macaques (102, 103).

Another regulatory target of estrogen and progesterone could be SERT, the serotonin reuptake transporter. Treatment with estrogen increased SERT mRNA in the dorsal raphe of rats (105) although the opposite effect (decreased SERT mRNA) was seen in macaques (106). The physiologic effect of transport regulation is unclear

however, since serotonin binding and uptake can be increased even the face of decreased SERT mRNA (106). In mice, a low estrogen state led to a reduced density of SERT in the hippocampus but a paradoxical increase in SERT activity (107). Other possible targets for protein regulation like the 5HT1A autoreceptor (108) or MAO-A (109) show responses to various combinations of estrogen and progesterone.

It can therefore be concluded that serotonin neurons have the basic properties necessary to respond to female sex hormones – they express the appropriate receptors and have the ability to regulate both mRNA and protein in response to these signals. It is equally clear however, that invoking sex hormones to account for the difference in the Pet-1 knockout mice requires that sex hormones be able to modulate serotonergic neural responses.

Accordingly, a wide range of functions in which serotonin is implicated have been shown to be modulated by female sex hormones (110). The most basic of these is a difference in the tonic activity of dorsal raphe serotonin neurons between the sexes. In male rats, serotonergic neurons were found to have an average firing rate 41% higher than normal and ovariectomized females that was *not* correlated with circulating levels of progesterone and estrogen. During pregnancy, the basal firing rate of the females increased up to 136%, and this rise and subsequent fall late in pregnancy was closely reflected in the circulating progesterone level (111). Further work by the same group established that these differences were due to a partial desensitization of the 5-HT1A receptors during pregnancy (112). Stimulation of the basal firing rate was achieved with intra-cerebral ventricular DHEA, progesterone, and its metabolites in the females (113), but these effects were not seen in males. Instead, the baseline rate in both males and

females was modulated by testosterone as well as with estrogen (which lies downstream from testosterone in metabolism) (114).

It would be imprudent to attempt to summarize all other actions of female sex hormones on the serotonin system here, suffice it to say that their ability to act on it is well-established (110).

The specific function in question here is the role of sex hormones on the serotonin system as it pertains to breathing. There is not much experimental evidence in this area, apart from the current study, the only work to address this question measured serotonin levels in respiratory nuclei in male and female rats throughout their estrus cycle (38). Females were found to have significantly higher levels of serotonin in the hypoglossal nucleus than the males, but equal levels in the phrenic nucleus. The serotonin levels in the different nuclei changed throughout the estrus cycle, with interesting results. In the hypoglossal nucleus, the changes in serotonin levels closely mirror plasma estrogen, while in the phrenic nucleus serotonin levels mirrored serum progesterone. These results are consistent with the current study, as downstream targets would be expected to show increased level of serotonin as the HCVR is influenced by hormonal status.

Given the enormous amount of evidence that links the serotonergic system with female sex hormones any evidence that the knockout phenotype of the Pet-1 male mice is altered by estrogen and progesterone must be taken seriously. The question remains however, if it accepted that estrogen and progesterone restored some of the remaining serotonergic function, why did they not restore all of the difference between the male and female Pet-1 knockout mice?

It is certainly possible that a higher dose of estrogen and progesterone could have shown a larger impact on the ventilatory response, but this would not have addressed the phenotypic difference between the sexes. Ovariectomy in the female mice would also have addressed this question, but is far more invasive than hormone supplementation in the males. Given the results of this study it is a logical follow-up experiment.

The most obvious explanation for the results is that acute supplementation with estrogen and progesterone in male mice cannot replicate the influence of these hormones throughout the neural and physiologic development of the female mice. There is support for this finding in studies of the sexual dimorphism of the serotonin system, where early in rat development (day E14) estrogen has effects on neurite growth (115). Within the second postnatal week, there are already sex differences in the serotonergic system in rodent brains (116). Klink et al hypothesize that the difference in the dorsal raphe basal rate response to estrogen and progesterone is a gender-dependent trait, as the ovariectomized female mice do not take on the male phenotype (111). If the difference seen in the female and male Pet-1 knockout mice is due to this sort of gender-dependent developmental mechanism then no amount of hormone supplementation would be able to fully restore the male phenotype.

In conclusion, the present study reports that male Pet-1 knockout mice have a variable ventilatory deficiency in response to a normoxic hypercapnic challenge, with a decreased HCVR at lower CO_2 exposures. This deficiency is partially reversed by supplementation with physiologic levels of female sex hormones, suggesting that the function of the remaining serotonergic neurons can be boosted by estrogen and

progesterone and providing a partial explanation for the sparing of the female Pet-1 knockout mice hypercapnic ventilatory response.

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