The ventilatory response of neonatal serotonin knockout mice when exposed to different concentrations of CO_2 and the implications for sudden infant death syndrome

A senior composition project presented by:

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Table of Contents

Abstract……4

I. Introduction
- A. SIDS: Factors and the Triple Risk Model……5
- B. CO_2 Accumulation and Sleep Environment……6
- C. Abnormalities in the CO_2 Detection Pathway……6
- D. Serotonin and Ventilation……7
- E. Current Research on Serotonin and Ventilation……8
- F. Validity of Study……8

II. Methods
- A. Animals and Genotype……10
- B. Collecting Tail Samples and Genotyping……11
- C. Phenotypic Analysis……11
- D. Subject Groups……12
- E. Testing Set Up and Gas Delivery……12
- F. Protocol……13
- G. Statistical Analysis……14

III. Results
- A. Ventilatory Response to Control Air and Observed Trends……14
- B. Ventilatory Responses to 4% CO_2……15
- C. Ventilatory Response to 8% CO_2 ……16
- D. Figure of Triple Risk Model and Partial Body Plethsymography……18
- E. Figures of Ventilatory Responses to 0% CO_2……20
- F. Figures of Ventilatory Responses to 4% CO_2……23
- G. Figures of Ventilatory Responses to 8% CO_2……26

IV. Discussion ……29

V. Reference ……33

VI. Appendix……35

Abstract

The accumulation of CO_2 around an infant's head is a major risk factor associated with SIDS. Serotonin imbalances in the brain have also been found in a high number of infants who succumb to SIDS, the exact correlation between SIDS and serotonin is still unknown. The purpose of this study is to investigate the ventilatory response of serotonin knockout mice (5-HTT) when exposed to CO_2. Two liters containing 14 mouse pups were split into two groups, serotonin knockouts or homozygous (-/-) and heterozygous (-/+). These mice were tested and weighed on days 2,3,5,7,9 and 11 after birth. The mice were placed in a partial body plethysmography chamber to detect the pressure changes during the ventilation cycle. Each mouse was exposed to a 0%, 4% and 8% CO_2 sequence over a period of 13 minutes. Using BioPac and Statview software the ventilatory response was recorded and analyzed to determine breathing frequency, minute ventilation and tidal volume. The results indicate that the serotonin knockout mice do not have a decreased CO_2 response resulting in a decrease in ventilation. This does not support the connection between SIDS and serotonin.

Introduction

Sudden Infant Death Syndrome: Factors and the Triple Risk Model

Sudden infant death syndrome is the unexpected, sudden death of a child under age 1 in which an autopsy is unable to find any other physiological cause of death (Krous et al. 2004). While there has been extensive demographic research on sudden infant death syndrome, much is still unknown. There are several factors that contribute to SIDS; such as, genetics, obesity, environment, infection, cardiac abnormalities, and brain neuron abnormalities (Thach 2008). The triple risk model demonstrates that SIDS only occurs if the infant is vulnerable, an exogenous stressor is present, and occurs during the first six months of life known as the critical development period (Kinney and Filiano 1994). Exogenous stressors would include sleeping position and sleeping environment. As illustrated by the triple risk model, sudden infant death syndrome may be a result of all three factors being present during a given time (Figure 1).

To date, the only preventative measures taken against SIDS are: putting the infant to sleep in a supine instead of the prone position and eliminating any pillows or heavy blankets from the crib (Thach 2008). Even with these measures, SIDS still remains the number one cause of death in developing countries for infants (Moon et al. 2007). The Back-to-Sleep campaign was created to educate parents worldwide about the dangers, causes, and precautionary measures that can be taken to prevent SIDS. After the Back-to-Sleep campaign was created in 1993 the incidence of SIDS dropped 40 percent (Anderson et al. 2005). For examples, in North Dakota, it was found that the reported rate of prone sleeping declined 72% in American Indian infants and 62% for Caucasian infants (McCulloch et al. 2000).

CO_2 Accumulation and Sleep Environment

There are several links between CO_2 accumulation around an infants head and SIDS, the theory being that when the CO_2 starts to build up due to sleep position or excessive blankets the infant is physiologically unable to detect the CO_2 change in the surrounding environment. The CO_2 concentration in expired end-tidal gas is about 4% (Chiodini and Thach 1993), but during the above mentioned conditions CO_2 levels have been recorded ranging from 3-6.4% (Malcom et al. 1994). If an infant is unable to detect this increase in CO_2 or unable to respond appropriately it can lead to abnormalities in ventilation and sensitivity to CO_2. (Malcom et al. 1994).

Abnormalities in CO_2 Detection Pathway

Abnormalities in olfactory CO_2 chemoreception and central chemoreception have both been identified as possible causes of SIDS (Mori et al 1990). Typically, olfactory chemoreception occurs in the nasal cavities as an odorant passes over the cilia and mucus of the nose a cascade of events occur that eventually leads to an action potential traveling to the medulla via the olfactory nerve, allowing for changes in ventilation (Stone et al. 2004). If the olfactory pathway is compromised, then this may lead to abnormalities in respiratory rate and ventilation. When a noxious gas such as CO_2 passes through the olfactory chemoreception or upper chemoreception there should be an initial decrease in ventilation (Stone et al. 2004).

There are also a sub-set of brainstem receptors that are able to sense CO_2, it has been hypothesized that some infants have less of these receptors or these receptors do not function

properly (Thach 2008). If such an infant was subjected to hypercapnic conditions, the increased CO_2 would not be detected and action potential will not occur to alert the medulla to act upon the parasympathetic nervous system and increase respiration; thus resulting in asphyxia (Nattie and Kinney 2002). Central chemoreception is located in the medulla oblongata, which controls respiration (Li and Nattie 1990). The medulla has control over forced expiration and the increase in rate for inspiration, and the pons activates the stimulus to take prolong inhalations (Stone et al. 2004). When the central chemoreception receives the signal from the olfactory system that there is a noxious gas in the environment such as CO_2 central chemoreception works through the medulla to activate the sympathetic nervous system to increase ventilation to try and blow off some of that noxious gas and maintain internal homeostatus (Stone et al, 2004).

Serotonin and Ventilation

Serotonin has come to the forefront of SIDS research because of its role in central chemoreception. Several infants who have succumbed to SIDS have been shown to have irregularities in the serotonin levels in there brain (Kinney et al. 2001). Serotonin, 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter (Adamec et al. 2006). It is involved in appetite, arousal, mood, thermoregulation, and pain control (Adamec et al. 2006). Serotonin by nature is not able to pass through the blood brain barrier; but can be transported in and out of the cells via serotonin transporters. These serotonin transporters are most concentrated in the raphe nuclei, which are vital in sending signals to the medulla (Ootuska and Blessing 2006). In some SIDS infants, the levels of serotonin in the extracellular fluid is higher

than normal meaning there is something wrong with the serotonin reuptake transporter, not enough serotonin is being take back into the presynaptic cells. In other cases, the serotonin levels in the extracellular space are lower than normal; this would indicate that the serotonin is being taken up too quickly in the presynaptic cells (Ootuska and Blessing 2006). It is still unclear which situation has contributed more to SIDS cases. In an infant with serotonin abnormalities, their initial response to having increased CO_2 in the environment would be to decrease their breathing, because the serotonin does not effect the olfactory chemoreception. When the signal travels from the olfactory system to the medulla and central chemoreception due to the serotonin transporter malfunction there will not be an activation of the sympathetic nervous system and there will not be an increase in ventilation. This will result in a infant who is actively re-breathing the increasing levels of CO_2 at a normal respiratory rate which will increase in affixation.

Current Research on Serotonin and Ventilation

One study found that it was clear that serotonin and the serotonin receptor are important for CO_2 chemoreception (Li and Nattie 2008). The 5-HT knockout mice in their study had a greatly reduced CO_2 response when compared to the wild type mice. Their results were able to show that the 5-HT knockout was the direct cause of the decrease in the CO_2 response (Li and Nattie 2008).

The study by Li and Nattie (2008) found that male rats have a greater diminished CO_2 response than female rats. In my study the male and female were placed into separate subgroups, but instead the mice were be divided up by wild type and serotonin knockout mice.

The differences in the CO_2 response of 5-HT knockout mice and wild type mice can possibly be explained by the existence of extra serotonin in the extracellular fluid or the presence of more serotonin in the brain tissue, and this is still unknown to researchers.

Validity of Study

I am interested in studying the effects of hypoxic conditions on the upper respiratory response in serotonin knockout neonatal mice. I hypothesize that there would be a decreased CO_2 response in the homozygous (-/-) mice when compared to wild type mice for both the four and eight percent CO_2 concentrations. The diminished response was expected to be more dramatic in the eight percent CO_2 exposure, and the CO_2 response should become less diminished as the serotonin pups age. This study would differ from previous studies because it is concentrated on the upper airway response. The homozygous (-/-) mice will have a decreased ventilation response to CO_2 when compared the heterozygous (-/+) mice, because the serotonin knockout makes

it impossible for central chemoreception to override the upper respiratory response and increase ventilation.

The reason for conducting this study is because most of the studies have been done on rats (Li et al 2006), piglets (Penatti et al. 2006) and rabbits (Ooutsuka and Blessing 2006), but little has been done with neonatal mice. Also, there are multiple studies that involve exposing the mice to hypoxic conditions and then physically analyzing the brains stem for investigation under a microscope without corresponding ventilatory data. There is little research involving

just the analysis of the breathing patterns when exposed to hypercapmic conditions, and this current study will help shed light on SIDS.

Methods

Animals and Genotyping

There were two groups of animals tested in my experiment; the serotonin knockout mice (homozygous (-/-)), and the control (heterozygous (-/+)). I did not test a separate liter of wild type mice due to the fact that some of the mice in the serotonin liter would be knockouts (homozygous) and some would be wild type (heterozygous) due to the genotypic make up of the parents. Given the time and resources the optimal experimental groups were two liters of the serotonin knockout mice (5-HTT knockout) with a total of 5 pups in the first liter and 9 in the second (Figure 2). I acquired one male and one female of the serotonin knockout strain and they were purchased from The Jackson Laboratory, and their stock name is B6.129 (Cg)-*Slc6a4*^{tm1Kpl}/J, and the stock number is 008355. These breeding pairs are sold as a heterozygous female and a homozygous male. The reason the female is heterozygous is because it was found that homozygous females do not take care of their pups. Both strains of mice were be housed in the Allegheny College animal room, with a twelve hour light cycle and a twelve hour dark cycle at a temperature of 21-24° C. The animals were fed mouse pellets and water ad libitum. Testing began after the pairs have been breed and the pups are born (18-21 day gestation period) (Hodges and Richarson 20008). All the mouse pups were tattooed with an ink dot on one or more paws to ensure identification within the litter

Collecting Tail Samples and Genotyping

When the subject liters of pups were born some of them were homozygous (-/-): serotonin knockout mice and some were wild type due to the parent's geneotype. Theoretically, given the original breeding pairs genotypes the liter could be half homozygous (-/-): serotonin knockout and heterozygous (-/+): control. This gives the study a blind study effect, and after all the testing was complete and the pups were about 35 days old a small section of their tail clipped off (.5 cm) and sent away to a genotyping company (Transnetyx) to discover the genotype of each mouse. The mice were anesthetized with the use of an 5% Isoflurane 100% oxygen chamber to calm them down before their tails were clipped. After the tail was cut and coagulation powder was administer to the bleeding tip of the tail to help stop the breeding and the mouse was placed in isolation until the bleeding stopped, approximately 5 minutes. The genotyping company results showed a total of 5 knockout (homozygous) mice, two knockout mice in the first liter both female, and three knockout mice in the second liter two females and one male.

Phenotype Analysis

During testing of the mice I kept a journal of the testability of each mouse; meaning how easy or difficult was the mouse to test (Appendix). I score each mouse on a scale from 0-5 on each day of testing. A score of 0 meant the mouse lay quietly in the chamber, it produced great breath peaks, was able to be handled without appearing visibly upset (did not bite, did not

squeak, or try to crawl away), and testing went very smoothly. A score of 5 meant the mouse would not settle down in the chamber, constant movement, tried to crawl out of the chamber, little usable breath peaks, was visibly upset when handled (biting, squeaking, and jumped out of my hands), and overall testing was not very successful. Each day of testing I observed the mice and gave them a score when they were done testing. I did this to see if there were any possible phenotypic differences between the homozygous (-/-) and heterozygous (-/+) mice, and I did not know the genotype of the mice at the time of testing therefore the results of my observations had to wait until after the mice were genotyped (Appendix).

Subject grouping

The subjects were placed into two groups; the 5-HTT knockout mice and the wild type mice (Figure 2). Then both groups were subjected to 3 different treatments; O%, 4%, and 8% CO_2 exposure for one minute.

Testing Setup and Gas Delivery

The mice were tested using head out plethysmography (Figure 3). A small plethysmography body chamber was used to initially test the neonatal mice, and then a larger body chamber was used depending on the size of the mouse. To calibrate the tidal volume in the different chambers a syringe was used to inject the chamber with a known amount of air. The body chamber was rested on a heating pad to enhance the comfort of the animal during the experiment. The mouse was resting in the prone position with its' head emerging through a latex collar; the cuff was secured to make an airtight chamber without obstructing the

breathing of the mouse. After the body of the mouse was secure in the chamber a CO_2 dispensing cone was placed over the head of the mouse, while its' head was resting comfortably on a sponge pillow. Several rubber weights and rubber bands were used to keep the chamber in place. The airflow through the chamber was controlled and independent of an increase or decrease in respiration. To record the concentration and the timing of the CO_2 a CO_2 analyzer was (BCI 9000) was connected to a port inside the chamber. BioPac computer software was used to interpret and analyze the signals from the CO_2 analyzer and pressure transducer.

Protocol

The mice were tested between the hours of 6pm through 11pm, to ensure consistency of the experiment and to ensure uniformity of the breathing patterns during their sleep cycle. The mice were tested on days 2,3,5,7,9 and 11 after birth. The air that was initially flowing through the chamber was control air (0% CO_2, 21% O_2 and balanced N_2) flowing at a rate of 1 mL/min (Stunden et al. 2001) (Figure 4). Once the mouse was placed in the chamber and the cone was placed over the head, the mouse was given time to acclimate to the chamber and resume normal breathing patterns. This took between 3 and 5 minutes depending on the age of the mouse (Coates and Silvis 1999). The mouse was then exposed to another form of control air to ensure the mouse had a steady breathing pattern and the change in air flow was not affecting the breathing pattern of the mouse. Then a cascade of switching from control air to different CO_2 concentrations followed: control air for 2 minutes, 4% CO_2 for 1 minute, control air for 2 minutes, 8% CO_2 for 1 minute, and control air for 2 minutes (Figure 4). The mouse was

then removed from the chamber weighed and placed back with the appropriate litter (Appendix). During testing phenotypic notes of mouse testability were taken on the behavior of the mouse: movement, was it easily agitated, crawling out of the chamber, calm, motionless (Appendix).

Statistical Analysis

The data were analyzed using BioPac and Statview computer programs. There were three measurements taken from the breathing pattern data. The last 10 seconds of control air, the first 10 seconds of exposure to CO_2 and the last 10 seconds of exposure to CO_2 (Figure 4). The frequency, tidal volume, average tidal volume, and minute ventilation were calculated and compared with 4% CO_2 and 8% CO_2 and then compared with the different days tested. The breathing frequency was determined by counting the total number of breath peaks in a ten second time interval. In BioPac, the tidal volume is equal to the area under the curve of each breath peak in the ten second time interval and was computed by BioPac. The minute ventilation was calculated by multiplying the breathing frequency by the tidal volume. The minute ventilation (air volume(mL)/min/gram) and tidal volume (air volume(mL)/breath/gram) were calculated by using the mouse weights. The values in the figures are means $\pm$ SEM. An ANOVA and Fisher's protected least significant difference test were be used to test the significance of the data, and the probabilities of $p<0.05$ were considered to be significant.

Results

Ventilatory response to control air and observed trends

The breathing frequency, tidal volume, and minute ventilation of the mouse pups in response to 0% CO_2 is shown in Figures 5, 6, and 7 respectively. In the heterozygous (-/+): control group there was a significant increase in breathing frequency in the last 10 seconds of exposure to 0% CO_2 on day seven (Fig. 5) The was a significant increase in the tidal volume in the last 1

seconds of exposure to CO_2 on day 5 in the wild type group (Fig. 6). In the homozygous (-/-): serotonin knockout group there was a significant increase in the first 10 seconds and the last 10 seconds on day 5 when exposed to 0% CO_2 (Fig. 6). In the homozygous (-/-): serotonin knockout group there was a significant increase in minute ventilation in the first 10 seconds and the last 10 seconds of exposure to 0% CO_2 on day 5 (Fig. 7). In the heterozygous (-/+): control group on day 2 there was a significant increase in the minute ventilation for the first and last 10 seconds of exposure to 0% CO_2. The breathing frequency increased (Fig. 5) and the tidal volume and minute ventilation decreased (Fig. 6 and Fig. 7) for days 2 through day 11 after birth for both heterozygous (-/+): control and the homozygous (-/-): serotonin knockout.

Ventilatory response to 4% CO_2

The breathing frequency in response to 4% CO_2 of 2, 3, 5, 7, 9, and 11 day old mouse pups separated in to heterozygous (-/+): control and homozygous (-/-): serotonin knockout groups is illustrated in Figure 8. There were no significant increases or decreases in breathing frequency on any day. When examining the 10 seconds before exposure to 4% CO_2 there is little variation. The breathing frequency did increase for both the homozygous (-/-): serotonin knockout and heterozygous (-/+): control groups from day 2 through 11 after birth.

The tidal volume in response to 4% CO_2 varied dramatically among the heterozygous (-/+): control and the homozygous (-/-): serotonin knockouts (Fig. 9). For the heterozygous (-/+): control group there was a significant increase in tidal volume of the last 10 seconds of exposure to 4% CO_2 for the heterozygous (-/+): control group and on days 2, 3, 5 (Figure 9). In the homozygous (-/-): serotonin knockout group there was a significant increase in tidal volume in the first 10 seconds of exposure to 4% CO_2 on days 2 and 5, and there was a significant increase in the last 10 seconds of exposure to 4% CO_2 on days 5 and 11 (Figure 9). The general trend for both groups from day 2 through 11 after birth was a gradual decrease in tidal volume except for day 5 on the homozygous (-/-): serotonin knockout there was a significant increase in tidal volume (Fig. 9).

The heterozygous (-/+): control had a gradual decrease in minute ventilation from day 2 through 11, and the homozygous (-/-): serotonin group had no obvious trend (Fig. 10). The last 10 seconds of exposure to 4% CO_2 was significantly higher in the heterozygous (-/+): control group for days 2, 3, and 5 (Fig. 10). The first 10 seconds of exposure to 4% CO_2 was significantly higher on day 3 (Fig. 10). There was an increase in minute ventilation on day 11 during the last 10 seconds of exposure to CO_2 in the homozygous (-/-): serotonin group (Fig. 10)

Ventilatory response to 8% CO_2

There was little significant difference in the breathing frequency for the heterozygous (-/+): control and the homozygous (-/-): serotonin knockout group (Fig. 11). The first 10 seconds of exposure to 8% CO_2 on day 11 in the heterozygous (-/+): control group was significantly higher (Fig. 11). In both the heterozygous (-/+): control and the homozygous (-/-): serotonin

knockout group there was a trend of increase breathing frequency from day 2 through 11 when exposed to 8% CO_2.

The tidal volume for the heterozygous (-/+): control and the homozygous (-/-): serotonin knockout does not seem to have a pattern to it and the data bars are extremely scattered, but there is a light decrease from day 2 through 11 (Fig. 12). In the heterozygous (-/+): control group there was a significant increase in tidal volume on days 2, 5, and 11 during the first 10 seconds of exposure to 8% CO_2 and the last 10 seconds of exposure to 8% CO_2 (Fig. 12). In the homozygous (-/-): serotonin knockout group the tidal volume was significantly higher on day elven during the first 10 seconds of exposure and the last 10 seconds of exposure to 8% CO_2 (Fig. 12).

The minute ventilation gradually decreases from day 2 through 11 in both the wild type and the serotonin groups, but the bars are very scatter (Fig. 13). In the heterozygous (-/+): control group there was a significant increase in the first 10 seconds of exposure to 8% CO_2 on day 11 (Fig. 13). In the homozygous (-/-): serotonin knockout group there is a significant difference in the first and last 10 seconds of exposure to 8% CO_2 on day 11 (Fig. 13).

Phenotypic Analysis

After receiving the genotype of each mouse I compared the results to my phenotypic journal of mouse testability. I had 5 mice that consistently score a 4 or 5 on each day of testing, and those 5 mice were all homozygous (-/-): serotonin knockout (Appendix).

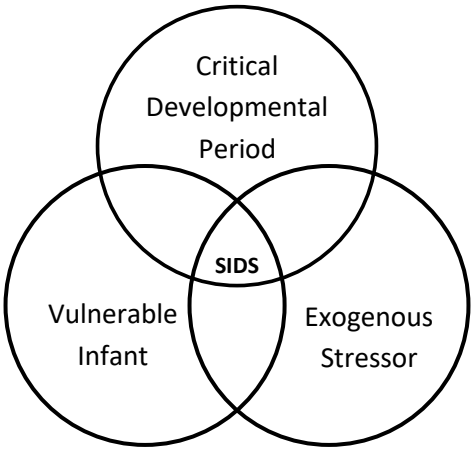


Figure 1: A Venn diagram depicting the triple risk model of SIDS (Filiano and Kinney 1994)

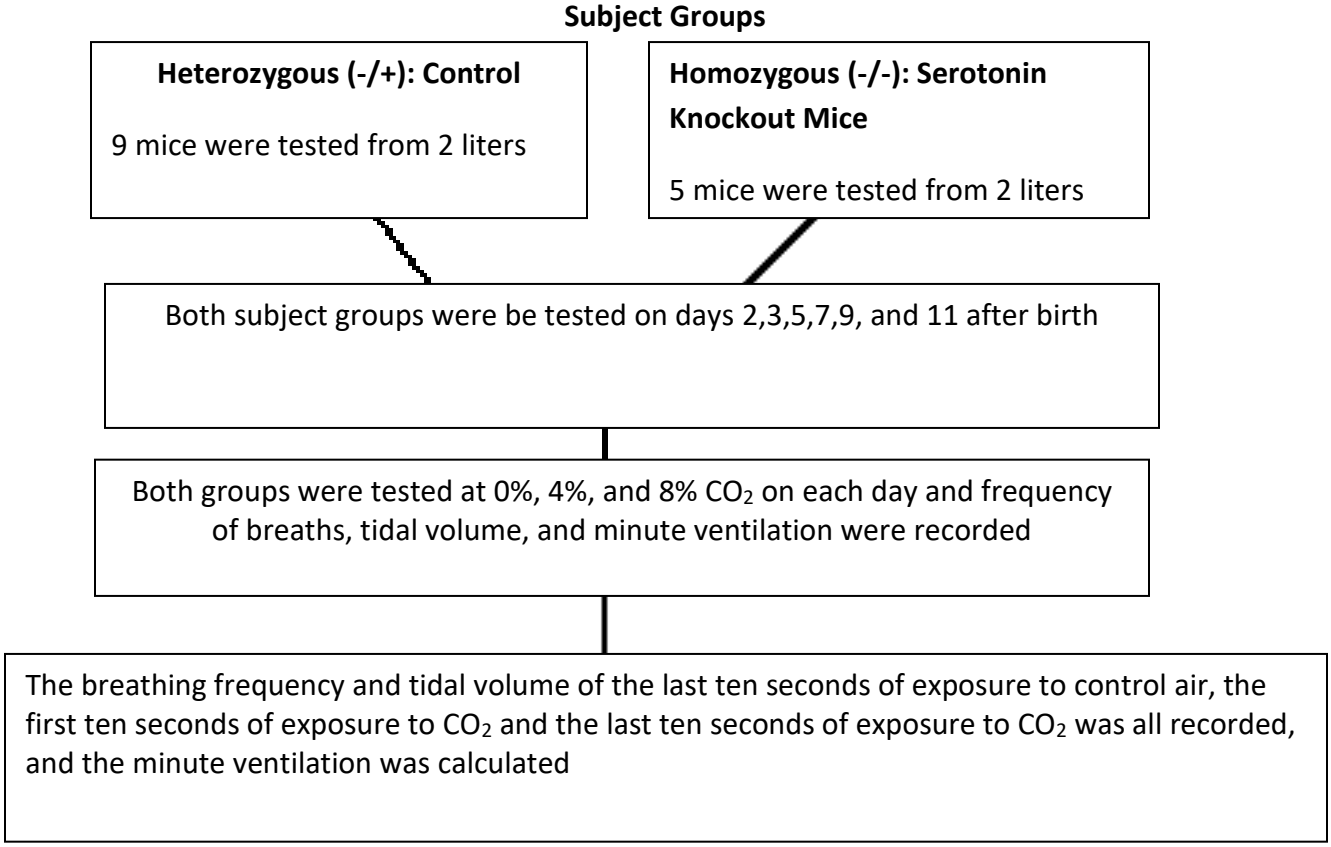


Figure 2: Breakdown of animals being tested, concentration of CO_2, and explanation of recording cascade

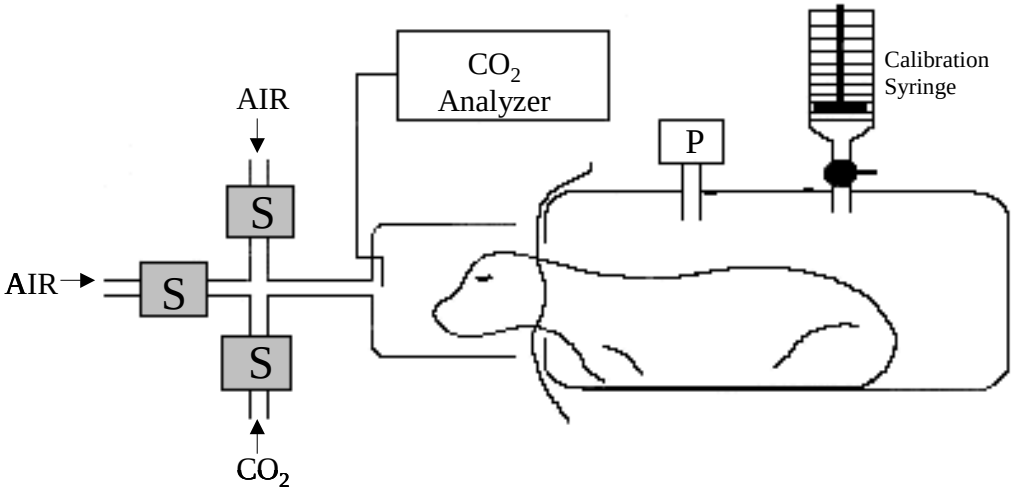
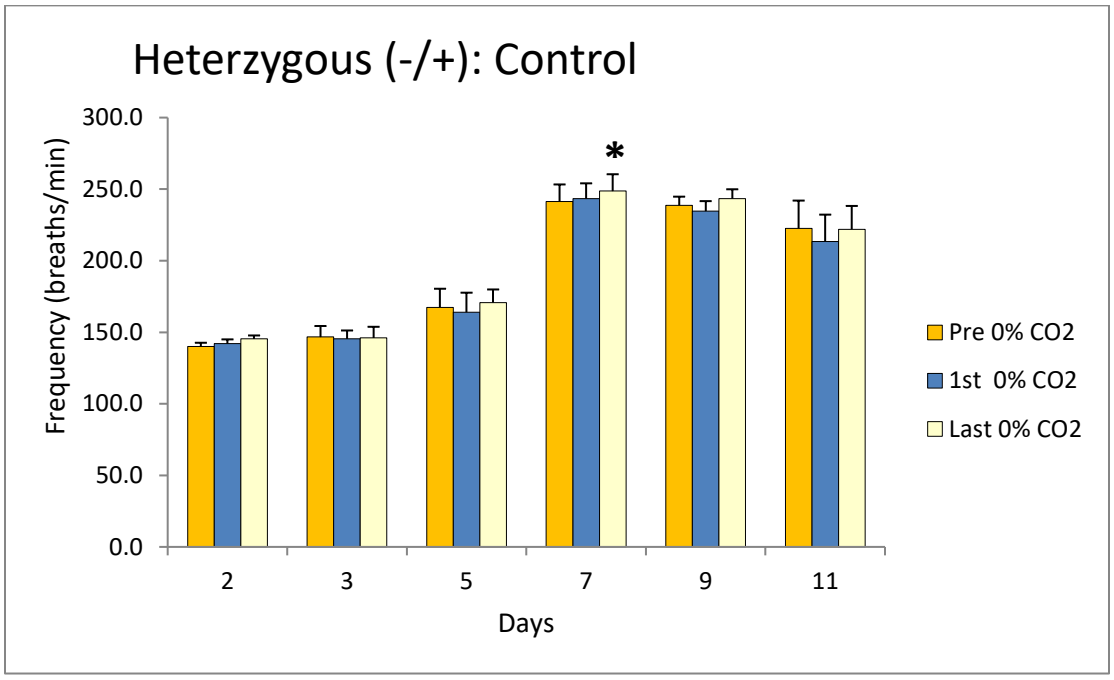


Figure 3: Head out plethysmography chamber containing a mouse pup used to deliver different concentrations of CO_2. S=solenoid and P=pressure transducer (Coates and Silvis 1999)

Patterns of CO_2 and control air exposure							
Length of Time	3-5 minutes	1 minute	2 minutes	1 minute	2 minutes	1 minute	2 minutes
Type of Air/CO_2	Air 2 Control air	Air 3 Control air	Air 2 Control air	4% CO_2	Air 2 Control air	8% CO_2	Air 2 Control air
Components of Air	0% CO_2, 21% O_2 and balanced N_2	0% CO_2, 21% O_2 and balanced N_2	0% CO_2, 21% O_2 and balanced N_2	4% CO_2, 21% O_2 and balanced N_2	0% CO_2, 21% O_2 and balanced N_2	8% CO_2, 21% O_2 and balanced N_2	0% CO_2, 21% O_2 and balanced N_2

Figure 4: The cascade of CO_2 and control air that the mice were exposed to


Heterzygous (-/+): Control
Frequency (breaths/min)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
*
2
3
5
7
9
11
Days
Pre 0% CO2
1st 0% CO2
Last 0% CO2

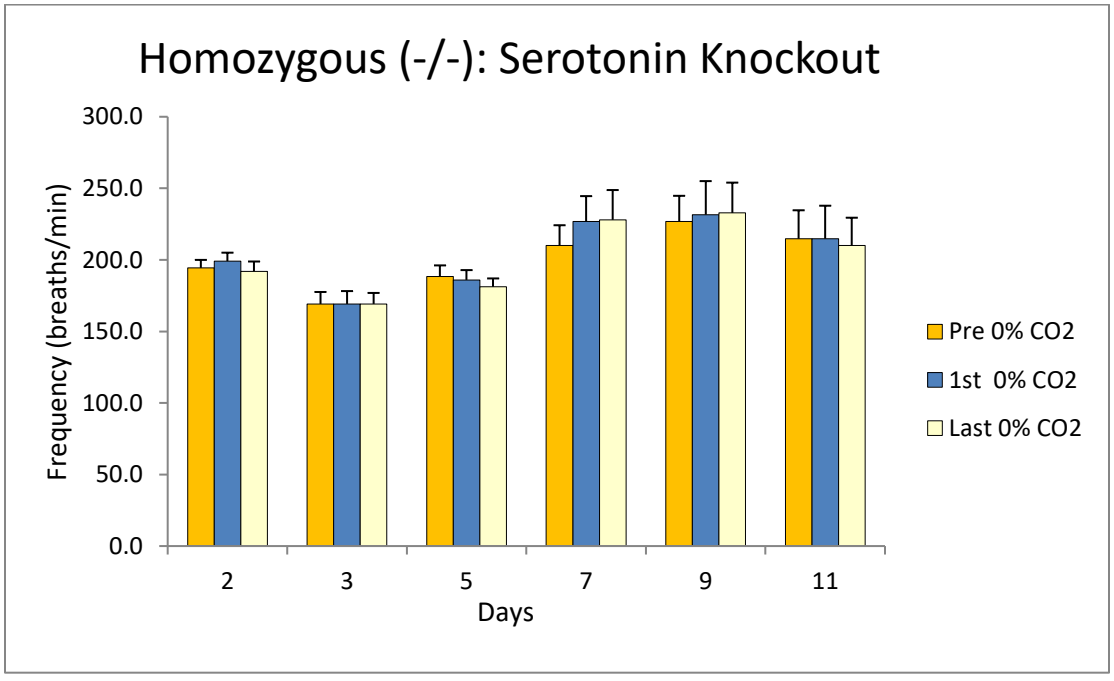
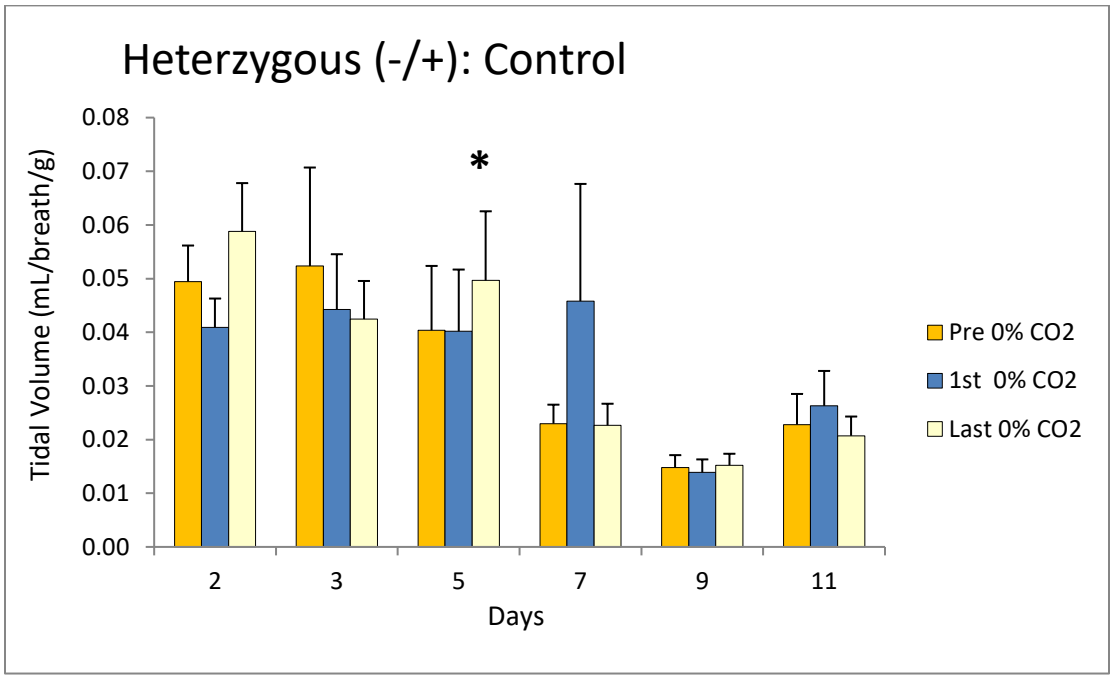

Homozygous (-/-): Serotonin Knockout
Frequency (breaths/min)
300.0
250.0
200.0
150.0
100.0
50.0
0.0
2
3
5
7
9
11
Days
Pre 0% CO2
1st 0% CO2
Last 0% CO2

Figure 5: Breathing frequency in response to 0% CO_2 of 2, 3, 5, 7, 9, and 11-day old mouse pups separated into two groups: heterozygous (-/+): control, and homozygous (-/-): serotonin knockout. The 0% CO2 was delivered for 1 minute and was divided into the 10 seconds prior to 0% CO_2 exposure (gold), the first 10 seconds of 0% CO_2 exposure (blue), and the last 10 seconds of exposure to 0% CO_2 (yellow). Asterisks show the significance ($p<0.05$) from pre CO2 values, the values are means ± SEM.



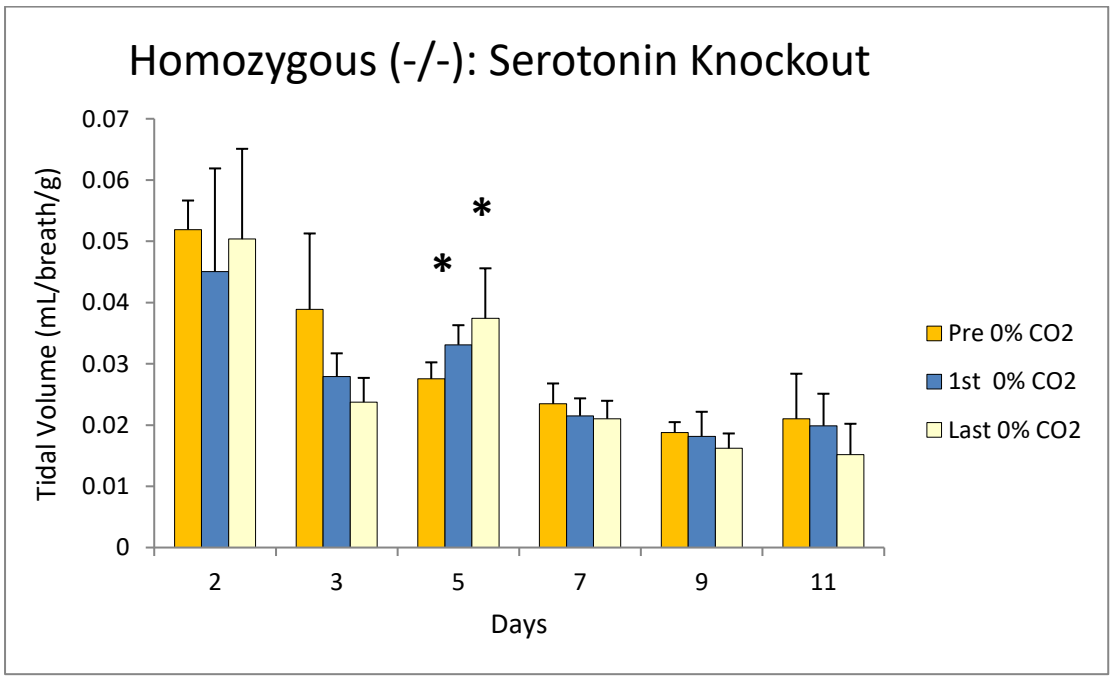
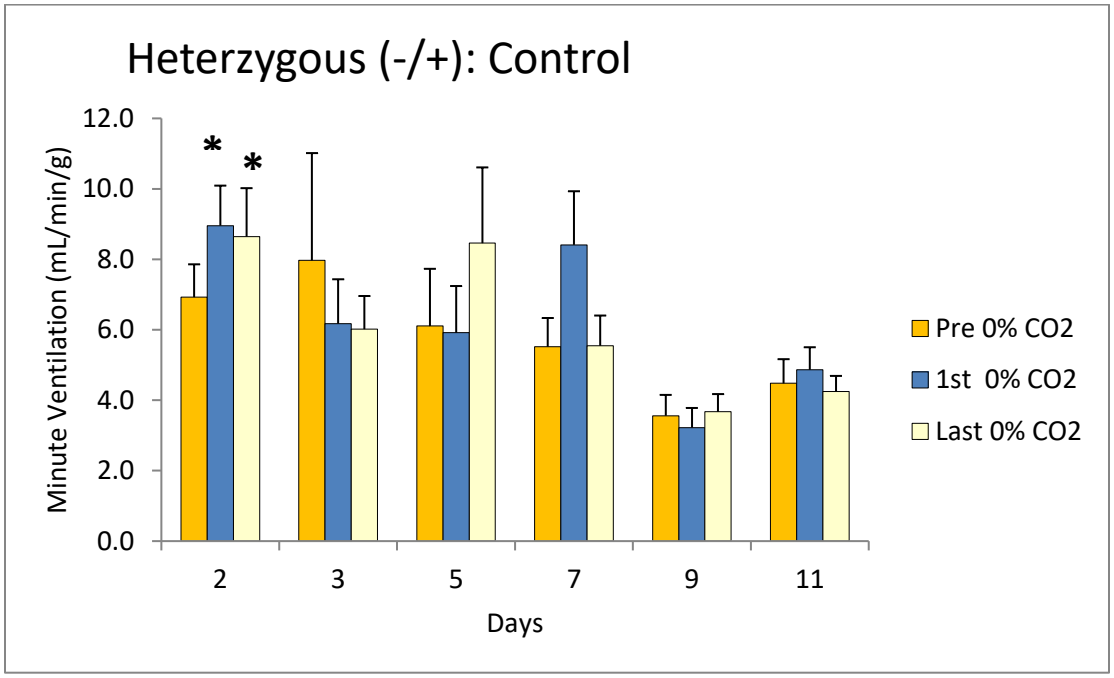


Figure 6: Tidal volume is response to 0% CO_2 of 2, 3, 5, 7, 9 and 11 day old mouse pups. Refer to Fig. 5 for definitions of asterisks and bars.



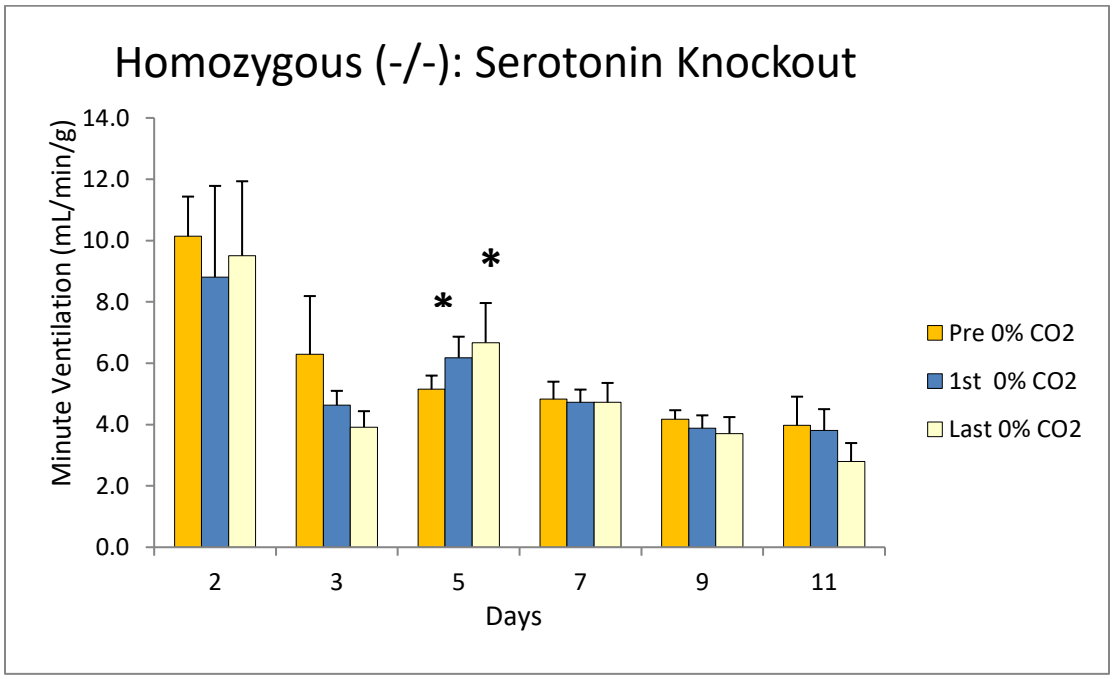


Figure 7: Minute ventilation in response to exposure to 0% CO_2 of 2, 3, 5, 7, 9, and 11 day old mouse pups. Refer to Fig. 5 for definitions of asterisks and bars.

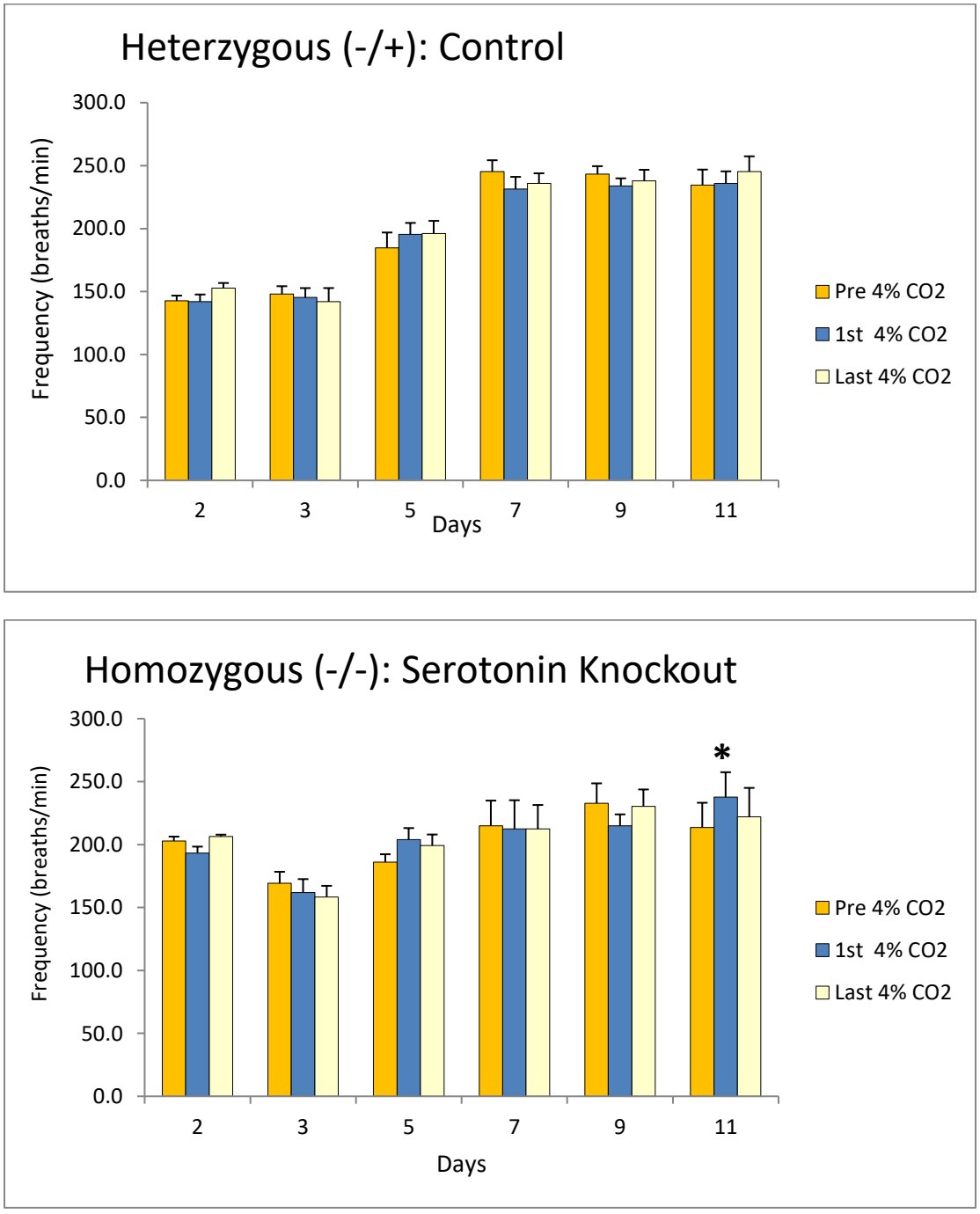
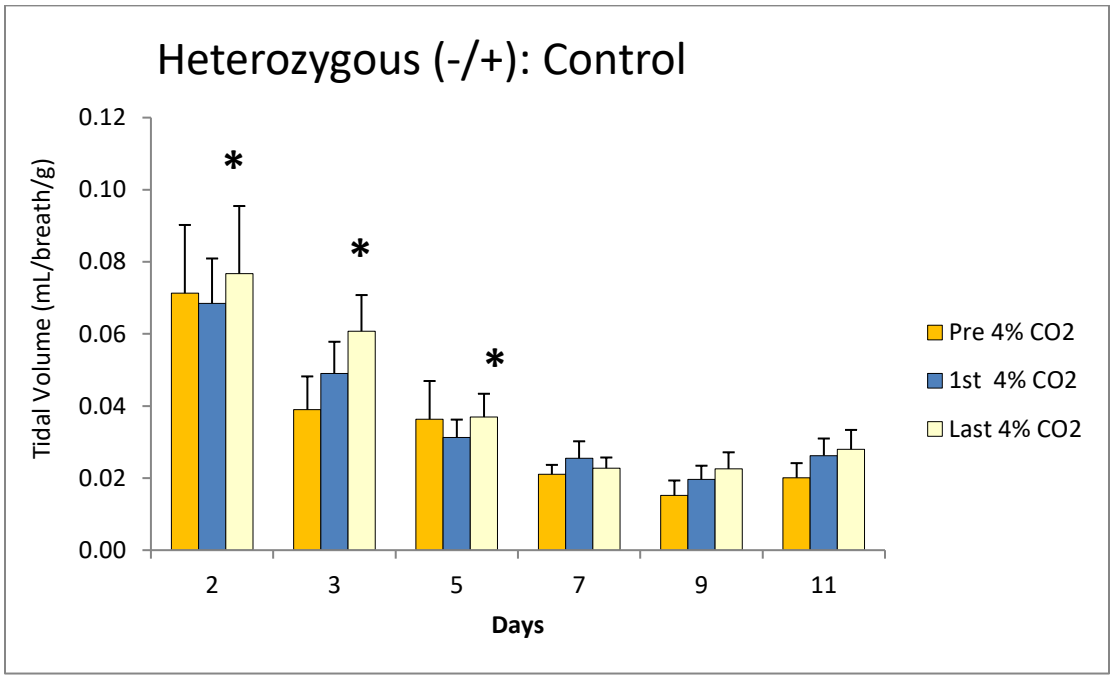


Figure 8: Breathing frequency in response to 4% CO_2 of 2, 3, 5, 7, 9, and 11-day old mouse pups separated into two groups: heterozygous (-/+): control, and homozygous (-/-): serotonin knockout. The 0% CO2 was delivered for 1 minute and was divided into the 10 seconds prior to 4% CO_2 exposure (gold), the first 10 seconds of 4% CO_2 exposure (blue), and the last 10 seconds of exposure to 4% CO_2 (yellow). Asterisks show the significance ($p<0.05$) from pre CO2 values, the values are means ± SEM.



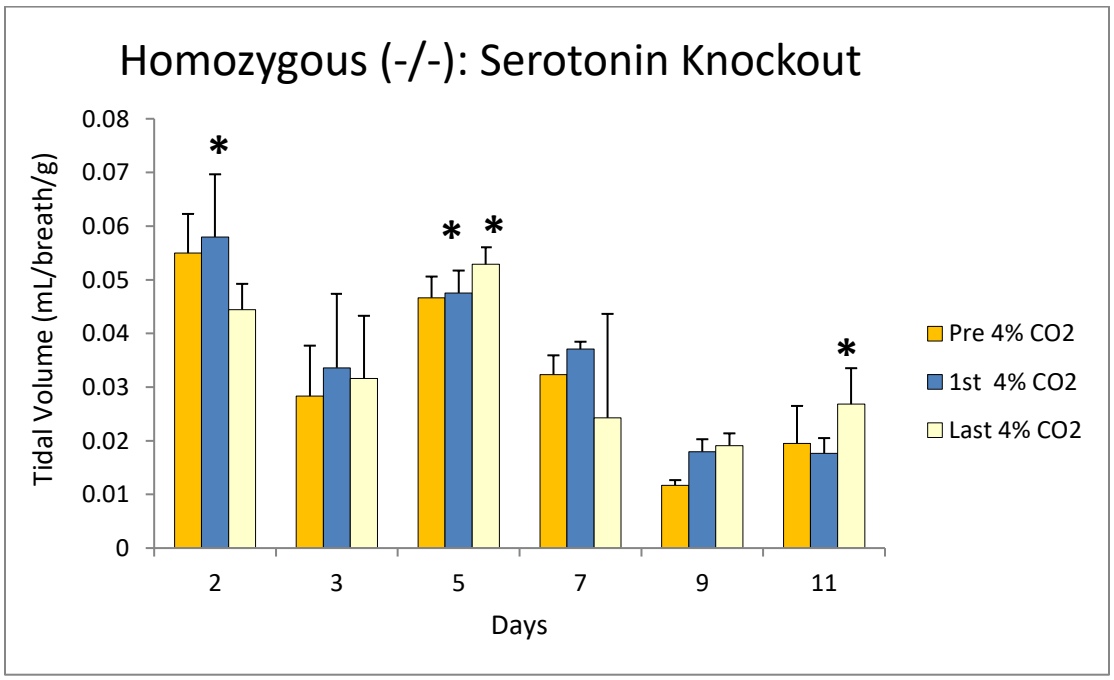
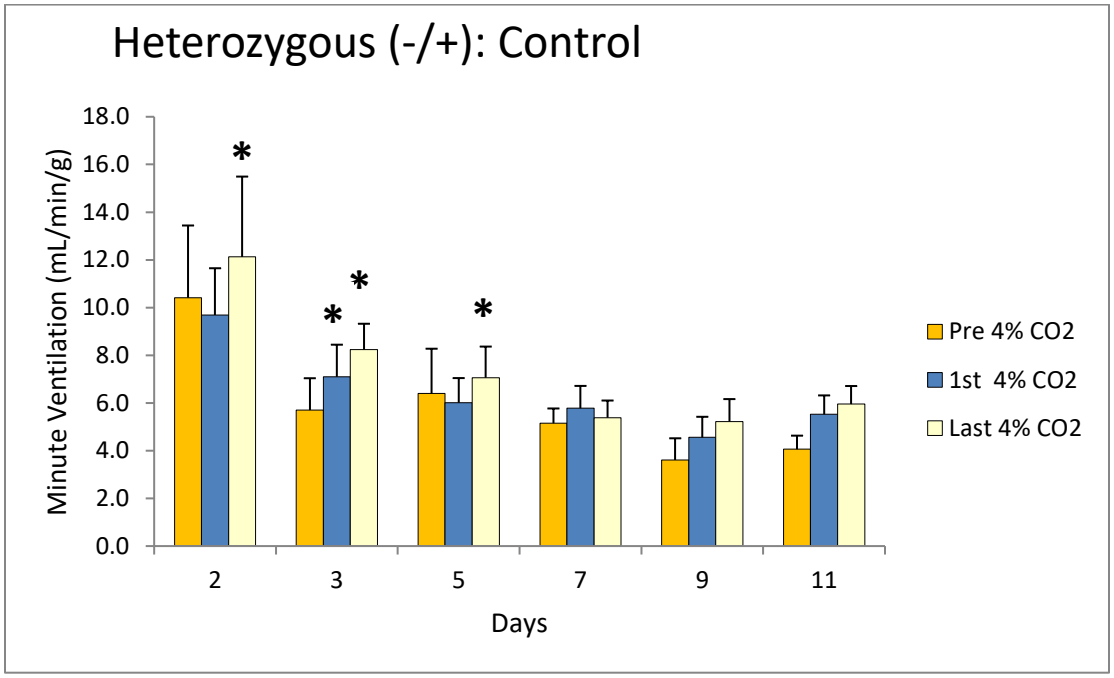


Figure 9: Tidal volume is response to 4% CO_2 of 2, 3, 5, 7, 9 and 11 day old mouse pups. Refer to Fig.8 for definitions of asterisks and bars.



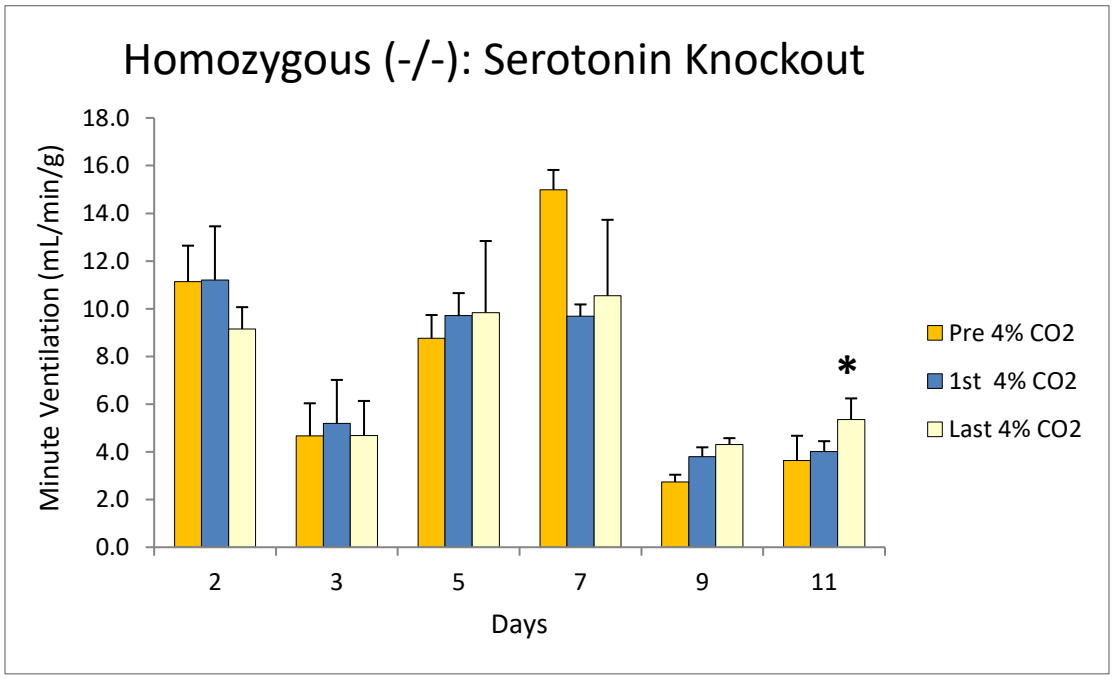
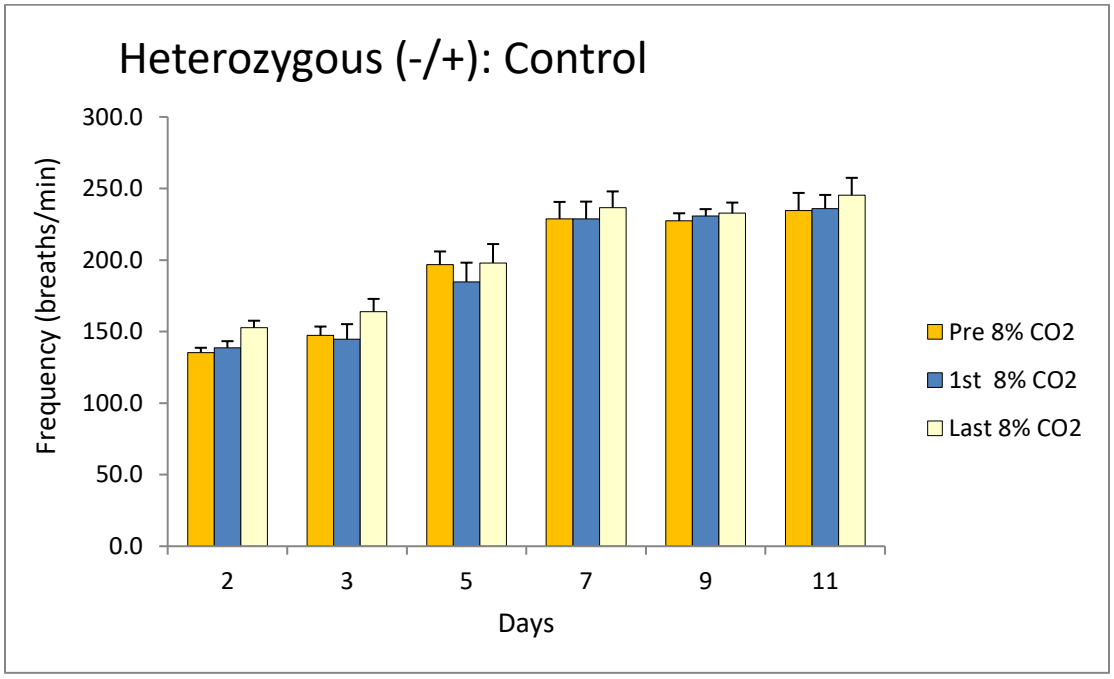


Figure 10: Minute ventilation in response to exposure to 4% CO_2 of 2, 3, 5, 7, 9, and 11 day old mouse pups. Refer to Fig. 8 for definitions of asterisks and bars.



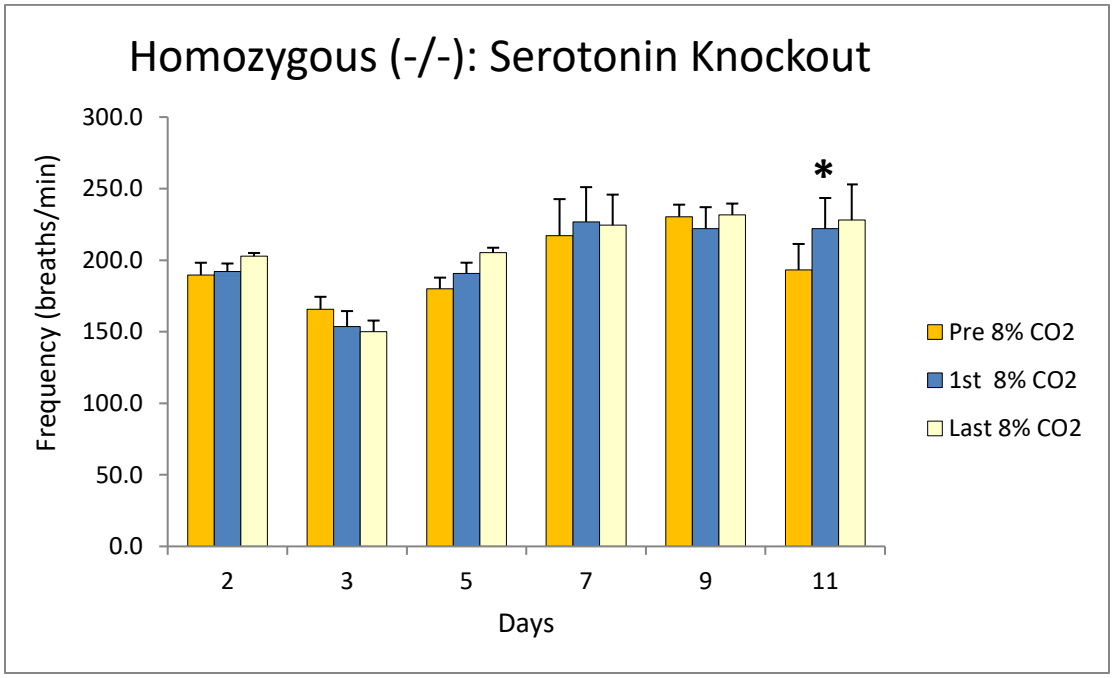
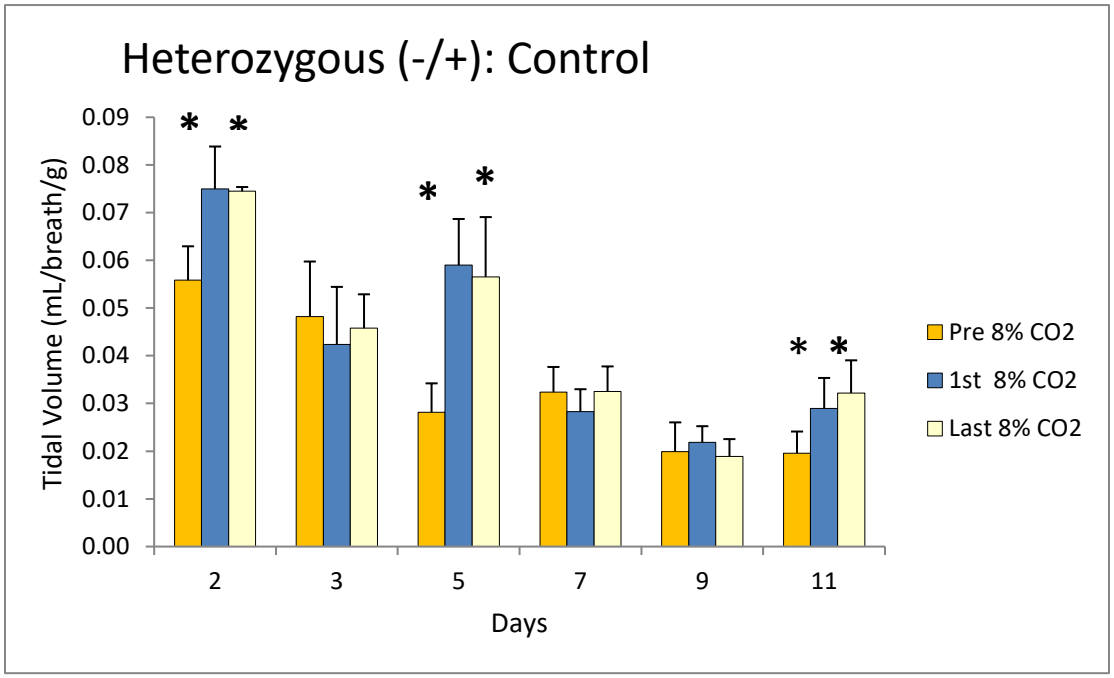


Figure 11: Breathing frequency in response to 8% CO_2 of 2, 3, 5, 7, 9, and 11-day old mouse pups separated into two groups: heterozygous (-/+): control, and homozygous (-/-): serotonin knockout. The

8% CO2 was delivered for 1 minute and was divided into the 10 seconds prior to 8% CO_2 exposure (gold), the first 10 seconds of 8% CO_2 exposure (blue), and the last 10 seconds of exposure to 8% CO_2 (yellow). Asterisks show the significance ($p<0.05$) from pre CO2 values, the values are means + SEM.



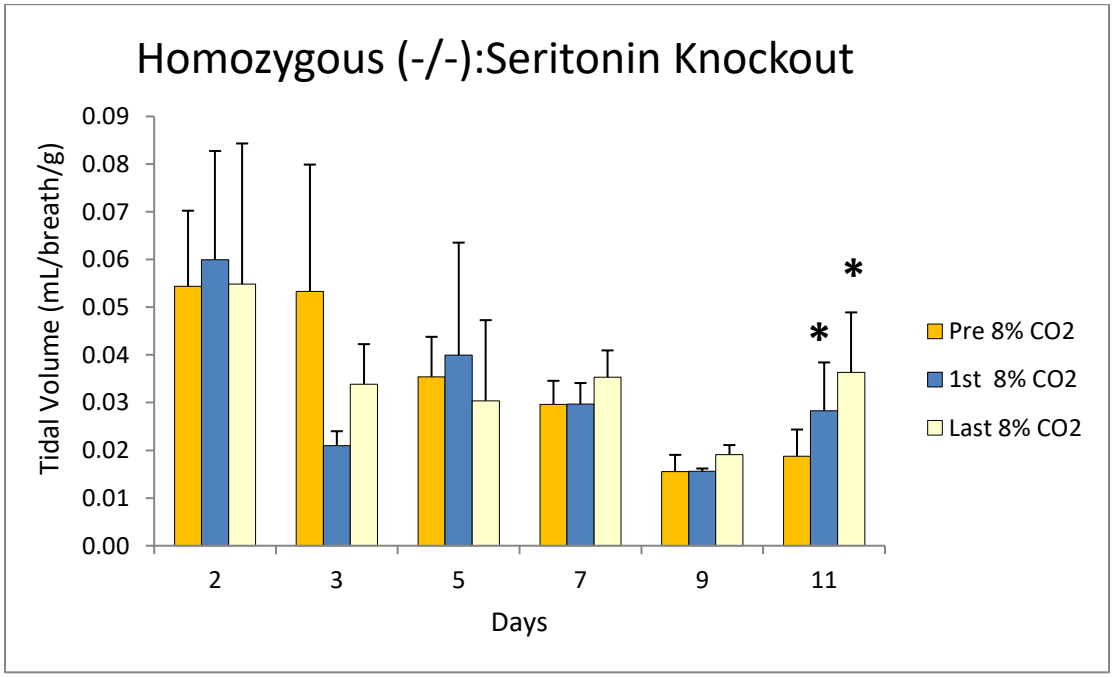


Figure 12: Tidal volume is response to 8% CO_2 of 2, 3, 5, 7, 9 and 11 day old mouse pups. Refer to Fig.11 for definitions of asterisks and bars.

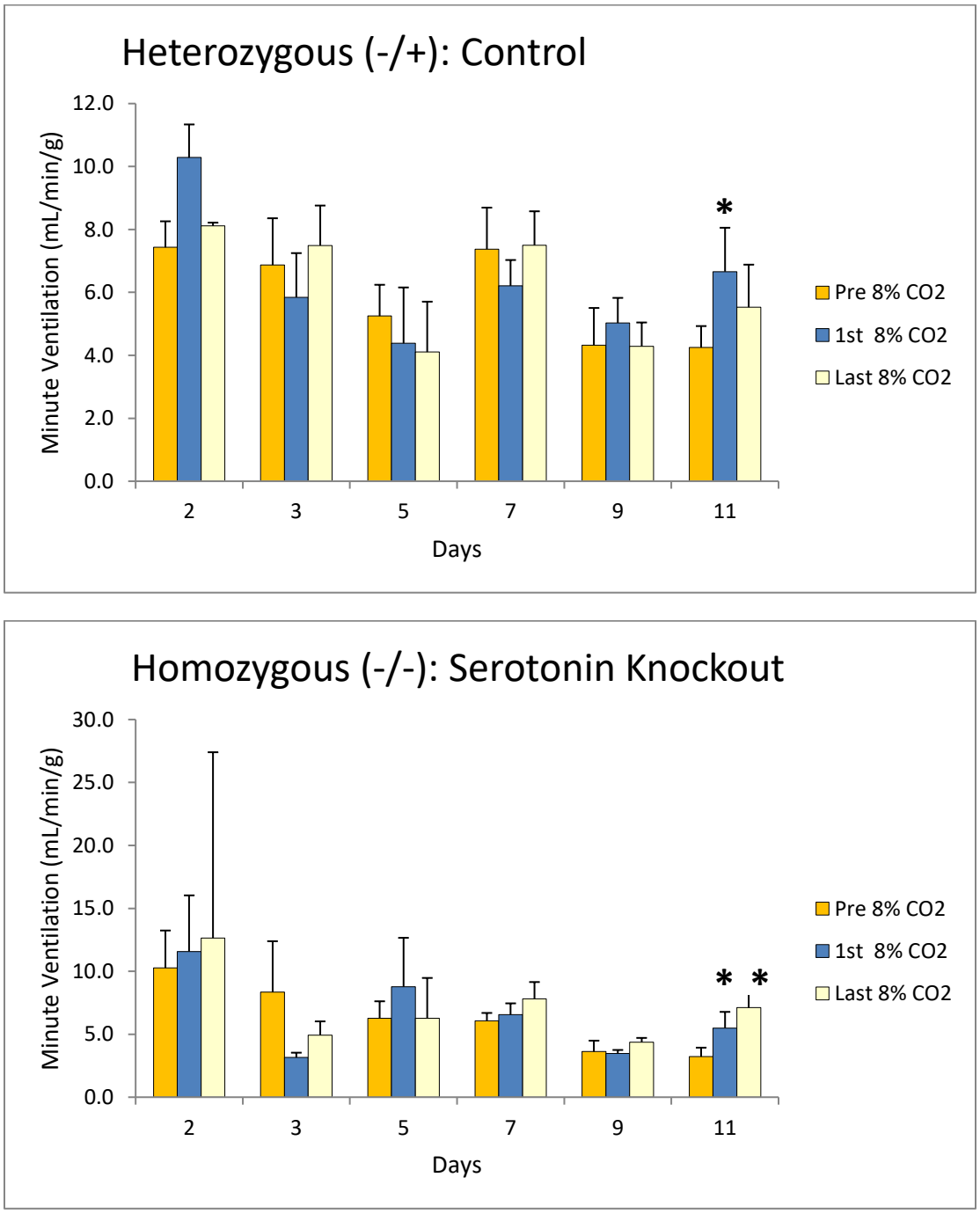


Figure 13: Minute ventilation in response to exposure to 8% CO_2 of 2, 3, 5, 7, 9, and 11 day old mouse pups. Refer to Fig. 11 for definitions of asterisks and bars.

Discussion

The current study examined the effects of CO_2 on ventilation of heterozygous (-/+): control mice and homozygous (-/-): serotonin knockout mice. This study was relevant to SIDS because the serotonin knockout has been found in many infants who have succumbed to SIDS. The original hypothesis was that there was going to be a decrease in the CO_2 response in the homozygous (-/-) mice when exposed to CO_2 and that the diminished response would be more dramatic when exposed to the 8%. The results of this study suggest that the CO_2 response is not diminished in the serotonin knockout mice and the concentration of the CO_2 do no show any significant differences.

To determine the normal ventilatory changes that occur as the mice age the breathing frequency, tidal volume and minute ventilation were all examined during exposure to 0% CO_2. There were almost no significant changes in the breathing frequency, tidal volume, or minute ventilation (Figs. 5, 6, 7). This verifies that the mice were not responding to the differing air flow changes that occur when switch between 0%, 4% and 8% CO_2.

The breathing frequency during the control air increased slightly from day 2 to day 11 for both the heterozygous (-/+): control and the homozygous (-/-): serotonin knockout mice suggesting that as the mice age they are taking more breaths per minute (Fig. 5). In other ventilatory studies it has been found that breathing frequency was shown to increase on day 1 to day 7 after birth (Li and Nattie 1990). Then, by day 12 average breathing frequency had

decreased from day 8, this suggesting that breathing frequency does not continue to increase with age (Rupert 2003).

In this study, the tidal volume decreased for both the heterozygous (-/+): control and the homozygous (-/-): serotonin knockout mice at 0%, 4%, and 8% CO_2 (Figs. 6, 9, 12). The tidal volume was calculated using each mouse pups weight, and mouse pups grow extremely quickly the first week of life. The mouse was expiring less volume pre breathe pre gram, which indicates that the decrease in tidal volume is due to an increasing weight in the mouse which is age dependent.

The minute ventilation was constant and only decreasing slightly from day 2 to day 11 for both the heterozygous (-/+): control and the homozygous (-/-): serotonin knockout mice at 0%, 4%, and 8% CO_2 (Figs. 7, 10, 13). This is due to the fact that the breathing frequency was increasing from day 2 to day 11 and tidal volume was decreasing from day 2 to day 11. They counterbalance each other resulting in a relatively constant minute ventilation.

The first ten seconds of exposure to CO_2 is the upper airway response, and the last ten seconds is when central chemoreception takes over and the ventilation increases (Eugenin et al. 2008). In the homozygous (-/-):serotonin knockout mice I expected to see a slight decrease in the breathing frequency in days 1-5 in the first ten seconds of exposure to CO_2. On days 7-11 in the first ten seconds of exposure to CO_2 I would expect there to be less of a decrease in breathing when compared to days 1-5. As a whole, when the breathing frequency of the homozygous (-/-): serotonin knockout mice is compared to the heterozygous (-/+):control mice the first ten seconds of exposure to CO_2 should have about the same values, because the serotonin knockout should not effect the upper airway CO_s response (Hodges et al. 2009). On

the last ten seconds of exposure to CO_2 the breathing of the homozygous (-/-): serotonin mice will not increase like it will in the heterozygous (-/+): control mice. The heterozygous (-/+): control mice's central CO_2 response is corrupted by the serotonin knockout, which will result in no increase of breathing from the first ten seconds of exposure to CO_2 to the last ten seconds of exposure to CO_2. This was not observed in this study. There was no evidence of there being any type of correlation between the first 10 seconds of exposure to CO_2 and the last 10 seconds of exposure to CO_2 in the serotonin knockout mice.

The results of the present study show that overall there is little significance to support the original hypothesis. Much of the homozygous (-/-): serotonin knockout data is very scattered and during data analysis there were a number of outliers that were hard to incorporate into the data analysis. This could be due to the fact that the homozygous (-/-): serotonin knockout mice are more prone to stress disorders and the testing experience may be something the mice struggle to cope with (Adamec et al. 2006).

A flaw in the study was the test subjects themselves. The homozygous (-/-): serotonin knockout group has a serotonin imbalance in their brain and does not only affect central chemoreception. Serotonin is also important in appetite, arousal, mood, thermoregulation, and pain control (Adamec et al. 2006). During testing there were phenotypic behavioral notes taken to see if there would be a phenotypic link between the homozygous (-/-):serotonin knockout mice, and these notes were compared to the genotyping results from the tail samples months later. Consequently, all of the knockout mice scored a 4 or 5 on almost everyday of testing. They would crawl out of the chamber, move during the recording, squeak constantly, and bite. The heterozygous (-/+): control mice usually settled down once they were in the

chamber and relaxed and there was no issue getting a good reading, but when the homozygous (-/-): serotonin knockout mice were constantly moving there was no choice but to accept mediocre raw data. If the homozygous (-/-): serotonin knockout mice would have been more cooperative then possible better breath peaks could have been obtained, and possibly show some of the expected significance.

In conclusion, this study was important to perform because serotonin does play a factor in SIDS, but it is unclear as to exactly what that role is. It may be that too much serotonin is being produced and cannot be taken up into the brain efficiently (Penatti et al. 2006). The serotonin receptor may not be processing the serotonin properly if at all (Nattie and Kinney 2002). There were some general trends among the homozygous (-/-): serotonin knockout mice, but they were consistent with the heterozygous (-/+): control trends. For both the homozygous (-/-): serotonin knockout mice and the heterozygous (-/+):control mice at 0%, 4% and 8% CO_2 there was an increase in breathing frequency from day 2 to day 11, decrease in tidal volume from day 2 to day 11, and a relatively constant minute ventilation from day 2 to day 11 (Figs. 5-13).

The present study examined the critical development period and the vulnerable components of the triple risk model of SIDS (Fig. 1). The development period was addressed by using neonatal mouse pups and testing on days 2 through 11 after birth, and the vulnerable component was the serotonin imbalance in the knockout mice. To obtain more conclusive evidence using the serotonin knockout mice possibly using a true wild type mice as the control and then compare it to the heterozygous (-/+) and homozygous (-/-) mice.

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Appendix:
Individual weights on days 2 through 11

Weights

Mouse						
Wild Type	**Day 2**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**
L1M2D2	1.14	1.61	2.25	3.61	4.41	5.80
L1M5D2	1.29	1.74	2.77	3.82	4.88	6.35
L1M6D2	1.48	1.98	3.60	4.32	5.25	5.82
L2M1D2	1.48	1.80	2.58	3.70	4.82	5.08
L2M3D2	1.54	1.59	2.71	3.85	4.89	5.20
L2M5D2	1.80	1.72	2.98	4.17	5.00	5.80
L2M6D2	1.58	1.93	2.68	3.77	5.10	5.60
L2M8D2	1.35	1.61	2.15	2.67	2.85	3.00
L2M9D2	1.66	1.92	2.90	4.08	5.40	5.70
Knockouts						
L1M3D2	1.30	1.86	2.73	3.68	4.52	5.78
L1M4D2	1.47	1.88	2.91	4.05	5.04	6.38
L2M2D2	1.66	1.81	2.66	3.40	4.21	4.64
L2M4D2	1.58	1.77	2.60	3.61	4.77	5.20
L2M7D2	1.60	1.93	2.61	3.51	4.80	5.20

Appendix:
Phenotypic Journal of the Testability of the Neonatal Mice

Testability of the Neonatal Mice in the CO_2 Chamber							
Scale: 0-5 (0 being an extremely testable mouse and 5 being an extremely untestable mouse)							
Mouse/Liter (M/L)	**Day 2**	**Day 3**	**Day 5**	**Day 7**	**Day 9**	**Day 11**	**Genotype**
M2L1	0	1	0	1	0	2	(-/+) Heterozygous
M3L1	**4**	**4**	**5**	**5**	**5**	**5**	**(-/-) Homozygous**
M4L1	**4**	**3**	**5**	**5**	**5**	**5**	**(-/-) Homozygous**
M5L1	0	0	2	2	1	0	(-/+) Heterozygous
M6L1	0	0	0	1	2	1	(-/+) Heterozygous
M1L2	2	0	1	1	1	0	(-/+) Heterozygous
M2L2	**3**	**4**	**4**	**5**	**5**	**5**	**(-/-) Homozygous**
M3L2	0	0	0	1	0	2	(-/+) Heterozygous
M4L2	**5**	**4**	**5**	**5**	**5**	**5**	**(-/-) Homozygous**
M5L2	3	4	3	3	3	4	(-/+) Heterozygous
M6L2	0	0	0	1	1	2	(-/+) Heterozygous
M7L2	**4**	**3**	**4**	**5**	**5**	**4**	**(-/-) Homozygous**
M8L2	0	0	0	0	1	1	(-/+) Heterozygous
M9L2	1	2	2	2	2	1	(-/+) Heterozygous