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THE EFFECTS OF BRONCHOCONSTRICTION AND ANXIETY ON LUNG FUNCTION

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ABSTRACT

Background: The goal of this study is to examine the effects of airway inflammation and bronchoconstriction on lung function in BALB/c mice. House dust mite (HDM) and methacholine are commonly used to model specific components of asthma in mice. HDM is used to induce chronic inflammation in the lungs and methacholine is used to induce bronchoconstriction, both important components in modeling asthma. In developing an animal model to study the connection between asthma and anxiety, it is important to establish an accurate dosage compensation when administering methacholine treatments to mice that have chronic airway inflammation as a result of HDM administration. Mice with chronic airway inflammation will be more sensitive to methacholine and will therefore show higher lung reactivity to the same dose of methacholine in comparison to mice that did not receive HDM administration. It is necessary to establish the correct dosage of methacholine for both treatment groups in order to compensate for this and mimic similar levels of bronchoconstriction.

In addition, this study will look at the connection between early life anxiety and lung function. Mice that show high levels of anxiety early in life could show increased sensitivity to methacholine. This study will address this possible connection in order to understand the potential importance of early life anxiety in asthmatics.

Methods: Periadolescent mice (n=21) were divided into four groups: 1) Mice exposed to HDM (HDM), 2) Mice exposed to Methacholine (Meth), 3) Mice exposed to both HDM and methacholine (HDM/Meth), and 4) a control group (Con). Plethysmography was used to monitor and record lung function as PenH values. Early life anxiety was measured using ultrasonic vocalizations (USV).

Results: It was shown that half a dose of methacholine for the HDM exposed mice produced similar increases in PenH values compared to mice that did not receive HDM. PenH values were

significantly greater for the mice that received the methacholine treatment in comparison to those that received saline, demonstrating the significant effect of methacholine on lung function. Mice that were characterized as high USV did not have greater PenH values in response to methacholine compared to low USV mice.

Conclusion: In the development of an animal model for asthma, mice pre-treated with HDM to cause lung inflammation only need a half dose of methacholine to cause lung function similar to a full dose in mice that are not pre-treated with HDM. For the USV analysis, mice that demonstrated high anxiety early in life did not respond to methacholine with greater PenH values compared to those that demonstrated low anxiety.

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Chapter 1

Introduction

Asthma is a leading chronic disease among children and adolescents in the United States. According to the Center for Disease Control, in a classroom of thirty students, on average, three of these students will have asthma (CDC, 2012). This leads to asthma being one of the leading causes of school absences across the country. Most importantly, the negative effects of asthma pose a serious risk to children and adolescents, some of which can be life threatening. The effects of asthma are as follows: shortness of breath, coughing, wheezing, tightness in the chest, and even death. These physiological effects are not the only direct effects of asthma, however. Recent studies have shown that adolescents who suffer from acute asthma attacks demonstrate an increased risk of anxious behavior that transcends into adulthood (Richardson et al, 2006). This has led to research into the potential for asthma to serve as a precursor to long-term mental disorders due to the stress and anxiety increase in patients with acute asthma attacks in adolescence. For example, a study found that 54% of 120 asthmatic patients had a history of psychiatric illness, including panic and major depression disorders (Afari et al, 2001). Therefore, it is essential to not only understand the physiological effects of asthma, but also the psychological effects that linger into adulthood. Understanding the causes behind the psychological effects could play a key role in preventing the development of psychiatric illness in children with asthma.

Epidemiological studies, like the one above, provide great insight into the correlation between asthma and anxiety. However, cause and effect cannot be determined from these studies. Due to the ethical and moral issues associated with testing humans, it is necessary to establish a quality animal model in order to mimic the effects of asthma in humans. One of ashtma's unique

characteristics is that symptoms begin to develop and surface early in life, so studying periadolescent mice is an effective way to model the complications associated with childhood development of allergic asthma. However, there are many facets of this complicated disease. Therefore, a single animal model will likely fail to address every component of the cellular and biochemical processes that take place in an asthmatic individual. Fortunately, mouse models have proven useful for modeling specific features of this chronic disease. Previous studies have demonstrated that mice that have undergone allergen sensitization and respiratory challenges have shown human-like asthmatic symptoms, such as wheezing, difficulty breathing, and airway sensitivity (Epstein, 2004). In our case, we will be focusing on the effects of a known bronchoconstrictor as well as a chronic inflammatory agent on the lungs of periadolescent mice.

As described above, much epidemiological research has been done on the correlation between asthma and anxiety, but little research has been done to examine this correlation from the opposite angle. However, one study examined if there is a connection between post-traumatic stress disorder (PTSD) and the diagnosis of asthma. The subjects of this study were individuals involved in the World Trade Center collapse on 9/11. The results suggested that individuals with probable PTSD were 1.65 times more likely to diagnosed with asthma, which was statistically significant after controlling for the effects of gender, ethnicity, income, smoking status, and dust exposure (Shiratori et al., 2012). My research will examine whether anxiety early in life plays a role in exacerbation of asthma symptoms later in life, which may worsen the anxiety-producing effects of asthma. If early life anxiety is a key factor in the exacerbation of asthma symptoms, it becomes even more important to catch and diagnose mental health disorders early in life.

Airway Inflammation and Bronchoconstriction

Human allergic asthma is a complicated disease that is characterized by chronic airway inflammation and bronchoconstriction. Exacerbations that are caused by chronic airway inflammation can be slowly reversed by anti-inflammatory agents over time (Bousqeut at al, 2000). Inflammation in patients with chronic asthma is a complex process that is not yet completely understood. It is known that all cells of the airways are activated and play a role in asthma (Bousquet at al, 2000). The response of airway inflammation in patients with asthma includes increased airway hyperresponsiveness. This tightening of the airway leads to shortness of breath, wheezing, and difficulty breathing. These effects usually do not persist for more than a day and are treated with short acting β_2-agonists (Bousquet et al, 2000). Both bronchoconstriction and chronic inflammation could be key factors in the correlation between asthma and mental health.

House Dust Mite (HDM) Extract

HDM was used in this study to model the allergic response that asthmatics experience. HDM is the most common airborne allergen, making it a suitable agent to model the chronic inflammation that is seen in the lungs of patients with asthma. HDM was chosen over ovalbumin for this study for several reasons. Ovalbumin has been used in multiple studies to model allergic asthma, but results have shown that it does not lead to chronic airway inflammation, rendering it ineffective as a model for what is seen in humans with asthma. In addition, exposure to ovalbumin has been shown to lead to inhalation tolerance, meaning that mice become desensitized to the effects of the agent and do not show chronic inflammation (Johnson et al., 2004). Chronic exposure to inhaled HDM has been shown to lead to increased activity of the cells

involved in airway inflammation. Also, HDM has been shown to cause airway remodeling in mice, leading to widespread goblet cell hyperplasia as well as significant amounts of collagen buildup (Johnson et al., 2004).

Methacholine and Bronchoconstriction

Methacholine was used in this study to cause bronchoconstriction in asthmatic mice and non-asthmatic mice. The methacholine challenge is used in the human population to diagnose asthma as well as determine the severity of asthma. Methacholine induces bronchoconstriction in both asthmatics and non-asthmatics by acting on the muscarinic (M_3) receptors on the smooth muscle of the airway. Histamine has also been used to cause bronchoconstriction and acts on M_1 receptors. Asthmatic patients have been shown to react more severely to this agent due to the chronic inflammation in the lungs. Therefore, bronchoconstriction will occur at a lower dose in patients with asthma during the administration of methacholine or histamine (Brannan, 2010).

Methacholine was chosen over histamine for this study due to results from previous studies. Martin et al compared the effectiveness of both methacholine and histamine as bronchoconstrictors. Their study found that methacholine was a more effective agent to induce bronchoconstriction in both humans and mice, whereas histamine did not have reproducible effects in mice (Martin et al., 1998). In addition, another study found that in a methacholine challenge with humans, both drugs had minimal side effects. However, histamine caused voice change in more subjects, making methacholine the safer and more effective agent for the methacholine challenge (Higgins et al., 1988). Both of these studies demonstrate the importance of methacholine in inducing bronchoconstriction in both asthmatics and non-asthmatics. However, it is most useful to our study because it does not cause airway remodeling as a result of chronic inflammation. Therefore, administration of methacholine can be used to examine the

psychological effects of asthma, as a result of labored breathing, in patients who have not been exposed to the agent that induces chronic airway inflammation.

Establishing Methacholine Dose

Mice treated with HDM will have significant inflammation in the lungs, mimicking the airway of a human asthmatic with chronic inflammation. Therefore, they will respond to methacholine at a lower dose than the group that was not exposed to HDM (Johnson et al., 2004). It is important to adjust for this difference in lung inflammation when exposing both groups to methacholine in the methacholine challenge. Determining the proper treatment dosage to give to each group is important in order to provide both groups a comparable labored breathing experience.

Plethysmography and PenH as a Measure of Lung Function

PenH, otherwise known as enhanced pause, is the measurement that was used in this study to quantify labored breathing as a result of bronchoconstriction. This was made possible by the use of the Buxco® whole body plethysmograph. Whole body plethysmography is advantageous for this study for multiple reasons. First and foremost, it is noninvasive and allows the mice to move freely, allowing for the monitoring of breathing in as natural of an environment as possible in the laboratory. As the animal breathes in the chamber, the volume changes and flows are monitored and related directly to thoracic movement. Most importantly, this machine allows for the administration of the bronchoconstrictor, methacholine, to observe and record the effects of this treatment in comparison to mice who receive saline treatment. Buxco's® whole body plethysmograph, as described on their website, is often used for asthma studies like ours

using the enhanced pause measurement, PenH. They define this measurement as an index of airway hyper-reactivity that can be used as an indicator of changes in airway resistance. An increase in PenH with increasing doses of methacholine indicates that the mouse is hyperresponsive to the increasing concentration of the bronchoconstrictor (Buxco®).

However, there has been much criticism surrounding the use of PenH as a measurement of airway resistance. Many papers have been released cautioning the use of this measurement. Lunblad et al (2007) strongly discourages the use of this measurement stating that PenH is based solely on a single time-varying signal, the pressure inside the chamber. They argue that two signals need to be measured in order to effectively measure airway resistance: pressure and either flow or volume. In addition, one study argues that there is no way to interpret quantitative changes in PenH. For example, a ten-fold increase in PenH clearly indicates more smooth muscle contraction in the airways than a doubling in PenH, but there is no way to determine exactly how much more (Mitzner, 1998). Therefore, this study, as well as others, suggests that PenH should only be used to represent some sort of nonspecific reflection of the pattern of breathing (Lunblad et al, 2007). Based on these strong criticisms, it is necessary to be cautious when using the PenH output as a direct measurement of airway resistance. However, we can safely use the PenH output to establish and recognize changes in the breathing rate of mice exposed to different concentrations of methacholine.

Ultrasonic Vocalizations (USV) as a Measure of Anxiety

As previously mentioned, there is a correlation between asthma and mental health. Multiple studies have demonstrated a positive effect between having asthma as a child and developing an anxiety disorder later in life. However, little research has been done to look at this association from the other side. Does early life anxiety correlate with an increased risk of

developing asthma as an adolescent? This is a question that can be addressed by observing and collecting early measurements of anxiety from periadolescent mice. USVs are a measure for examining initial levels of anxiety in mice immediately following birth. There are three known USV classes in mice. The class that was used in this study was isolation-induced USV, which occurs during the first two weeks of life and is a result of separation from the mother and littermates (http://www.avisoft.com/rats.htm). Therefore, increased isolation-induced USV can serve as a marker for early life anxiety. Most importantly, the USV marker could expose a correlation between early life anxiety and increased lung sensitivity as an adolescent which could worsen asthma symptoms.

Many previous studies have used isolation-induced USVs as a measure of a negative emotional state in both mice and rodents. There is a great deal of evidence supporting USV output as a measure of anxiety, but there is also criticism to this claim. One study, in particular, claimed that these vocalizations were simply a byproduct of a thermoregulation process. They hypothesized that the detected vocalizations were a result of the natural physiological response to the abdominal compression reaction, which is a maneuver that increases venous return to the heart (Blumberg et al., 2001). However, subsequent studies generated evidence weakening this claim. In particular, a study done by Takahashi et al (2009) presented strong evidence supporting USVs as a measure of a negative emotional state. The researchers treated pups with anti-anxiety drugs, specifically benzodiazepines and other compounds that are positive regulators of GABA receptors, and examined the effects on isolation-induced USV output. Benzodiazepines are prescribed to the human population for short term relief of severe anxiety. They found that pups treated with these anti-anxiety drugs displayed reduced USV output, which is strong evidence in favor of isolation-induced USV being a result of anxiety and not simply a physiological response to the abdominal compression reaction (Takahashi et al, 2009). This evidence is important to this

study because it allows us to use isolation-induced USV as a measure of early life anxiety with greater confidence.

Purpose and Hypotheses

The purposes of this study are to quantify the effects of both airway inflammation and bronchoconstriction on lung function. Since HDM causes inflammation, it is hypothesized that mice treated with HDM will show greater sensitivity to methacholine and therefore, respond with greater PenH values to the same dose given to mice not exposed to HDM. In order to control for this dosage effect, it is hypothesized that mice exposed to HDM will respond to half a dose of methacholine with similar PenH values as the mice not exposed to HDM who are given a full dose of methacholine. Therefore, it is hypothesized that mice treated with HDM-Meth with a methacholine dose that is half that of mice treated with methacholine only will have similar increases in PenH values during their time in the plethysmograph, which is important in order to control for a similar experience of an asthma attack in both groups.

A second purpose of this study is to examine the correlation between early life anxiety and lung function. It is hypothesized that mice that have high anxiety, measured by isolation-induced USV, will respond with greater PenH values when exposed to the same dose of methacholine as the low anxiety group. If this is the case, then asthma may be impacted by early life anxiety, which would be an important finding in terms of recognizing and treating anxiety disorders early in life in order to prevent the exacerbation of asthma symptoms later in life.

Chapter 2

Methods

Study Subjects

The BALB/c mouse strain was used for this study for multiple reasons. These mice have been used to model asthma in previous studies and have demonstrated greater levels of airway reactivity to methacholine (Geuders et al., 2009). In addition, these mice have been shown to react to house dust mite (HDM) with asthma-like symptoms, including inflammation and airway remodeling (Saglani et al., 2009). There were three litters of BALB/c mice, each of different size, that were used for this study. In total, 21 mice were used (a litter of 3 mice, a litter of 5 mice, and a litter of 13 mice). However, due to technical difficulties with the plethysmograph, data from every mouse could not be obtained. The mice were kept in a consistent environment throughout the duration of the study. This environment included 12:12 light schedule (lights off at 6:00 am EST) with temperature, humidity, and ventilation all controlled.

Study Design

HDM Treatment

The 21 mice were divided into four groups: 1) Mice exposed to HDM (HDM), 2) Mice exposed to Methacholine (Meth), 3) Mice exposed to both HDM and methacholine (HDM/Meth), and 4) a control group (Con). The protocols for HDM and methacholine administration are presented in Appendix A. HDM was administered three times per week to group 1 and group 3

via nasal inhalation during the first eight weeks after birth. Group 2 and group 4 were administered saline via nasal inhalation to control for handling and treatment of the HDM pups.

Methacholine Treatment

In order to expose the animals to methacholine, each animal was placed individually into the chamber of the plethysmograph machine. Following a three-minute acclimation period to allow the mouse time to adjust to the new environment, baseline readings of PenH values were collected and recorded for three minutes. Once baseline was established, each animal was administered a dose of saline via the nebulizer of the plethysmograph. Mice in group 2 and group 3 then received increasing concentrations of methacholine doses. In order to account for the increased sensitivity of the lungs of mice treated with HDM (Group 3), the dose of methacholine was halved for these animals in comparison to the dose given to group 2. A half dose was chosen for mice exposed to HDM due to previous research comparing PenH values versus concentration of methacholine for mice exposed to HDM and mice not exposed to HDM. This data showed that at intermediate concentrations of methacholine, PenH values were about two times greater for the group exposed to HDM (Ferrara et al., Figure 2-1). Group 3's increasing dosage concentration of methacholine was 3, 6, 12, 25 mg/ml. Group 2's increasing dosage concentration was 6, 12, 25, 50 mg/ml. PenH values were recorded during the administration of each dose. Significant bronchoconstriction was signified by both high PenH values as well as obvious signs of labored breathing, drooling, and inactivity. For humane reasons, animals were removed from the plethysmograph if their PenH readings were greater than 15 units or if they drooled for more than two minutes. Animals that did not receive methacholine treatment, those in group 1 and group 4, were administered saline throughout the duration of their time in the plethysmograph.

Figure 2-1:

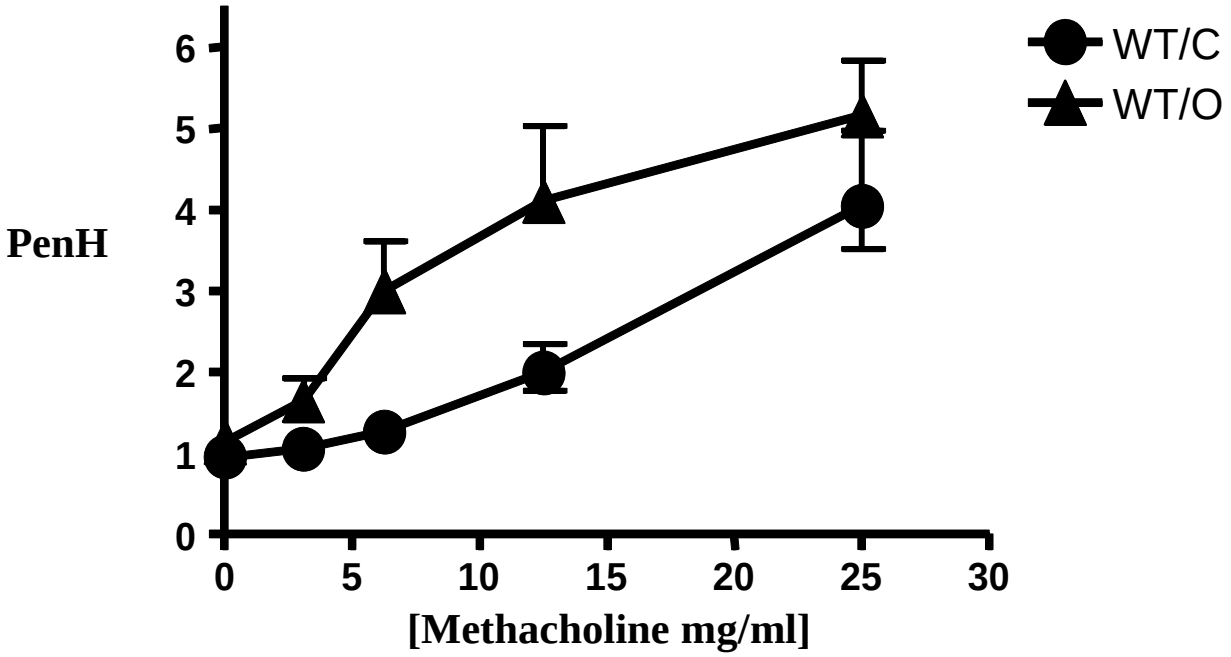

Figure 2-1. (a) Methacholine causes airway hyper-responsiveness (~PenH) in allergen-exposed (WT/O) and control (WT/C) BALB/c mice. This data showed that at intermediate concentrations of methacholine, PenH values were about two times greater for the allergen exposed group. Figure adapted from Ferrara et al., 2006.

Timetable

In order to mimic the symptoms and effects of childhood asthma in humans, both HDM and methacholine were administered to neonatal and periadolescent mice (Saglani et al., 2009). In order to maintain the identity of each mouse after birth, each mouse was marked with a different number with a permanent marker. This method was used until the mice were old enough to be ear-marked. HDM or saline treatments were administered 24 times between postnatal day (PD) 3 and 56. Methacholine or saline treatments were administered six times between PD 22 and 57.

This treatment regimen is shown in Figure 2-2. The mice were sacrificed in order to collect physiological samples on PD 139.

In order to record a known measure of separation anxiety early in life, very early in the study (PD 2-5) the ultrasonic vocalizations (USV) of each mouse were obtained. After HDM and methacholine treatments, behavioral tests of anxiety-like behavior were performed and outcomes were measured. However, these behavioral outcomes will not be discussed in this study.

Figure 2-2:

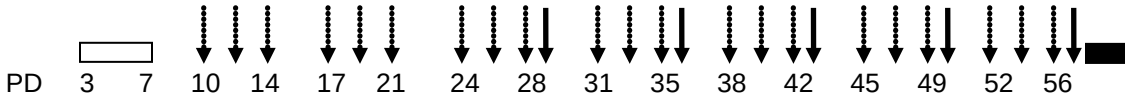

Figure 2-2. ***White bar indicates the neonate measure of anxiety-related behavior (ultrasonic vocalization) to document pre-asthmatic internalizing behavior***. Solid arrows indicate exposure to methacholine to cause labored breathing, dotted arrows indicate exposure to allergen [house dust mite (HDM) extract] to stimulate allergic inflammation. HDM exposure begins earlier than methacholine because several weeks of HDM exposure are required before significant inflammation occurs. Black bar indicates the late-adolescent circadian CORT rhythm measure.

PenH Analysis

The output from the plethysmograph was recorded onto an external hardrive in the procedure room of the Centralized Biological Laboratory (CBL). The data that were used for my study were the PenH output for baseline and the subsequent doses of saline (groups 1 and 4) or methacholine (groups 2 and 3). This output was transferred from the external hardrive onto Microsoft Excel for organization and data analysis. Average PenH values for baseline and the

subsequent doses were recorded for each animal. This was necessary because the plethysmograph records and collects values at many time points throughout the three-minute exposure to the methacholine/saline treatment. Due to technical issues with the plethysmograph, data were not recorded for every animal. Those with missing data were removed from the data set.

The average PenH values for each animal were collected and then averaged together with animals in their respective treatment groups. Plots of PenH versus concentration of methacholine were generated for each group at PD 22, PD 29, and PD 36. The same plots were obtained using data from individual animals to show how each animal responded independently to treatments over time.

USV Analysis

USV data was collected using a bat detector set to 65 kHz. Isolation-induced USV from periadolescent mice ranges from 50-80 kHz, so 65 kHz was chosen as a midpoint (Hofer et al, 2002). Pups were individually separated from their mother and placed into a separate cage where USV calls were tallied over a two-minute time period and then the pup returned to the cage with its mother. This was repeated for every pup. USV was collected over three consecutive days for each pup from PD 2 to 5. Data collection ended after PD 5 in order to begin HDM treatment. It was important to establish high and low USV callers prior to HDM treatment in order to have equal numbers of high and low callers in each group. Based on previous experience, USV calls tend to diminish around PD 8.

To determine whether to classify a pup as high or low, each pup was characterized as being above (high) or below (low) the median calling rate within its litter at each of the three time points (PD 2 to PD 5). High USV was classified as having at least two out of three USV values

above the median; low USV was classified as having at least two out of the three USV values below the median of the same sex litter.

Statistical Analysis

To verify that methacholine administration caused labored breathing, a repeated measures ANOVA was used to determine if methacholine exposure caused greater Penh values than saline. The factors in this analysis were dose/time (Doses 1-6) and methacholine exposure (methacholine vs. saline).

To test the dosage hypothesis, we ran a repeated measures ANOVA with the Methacholine only and HDM-Meth groups (Groups 2 and 3). The first factor in this repeated measures ANOVA was dose (Dose 1-6), second factor was group (Meth vs. HDM-Meth). For these analyses we wanted to compare the half dose for the HDM-Meth group with the full dose for the Meth group to verify that there was no statistical difference between the two. The doses for each group were as follows: Group 3's increasing dosage concentration was 3, 6, 12, 25 mg/ml. Group 2's increasing dosage concentration was 6, 12, 25, 50 mg/ml. We expect that dose should affect PenH values. As the dose of methacholine is increased, there should be a significant increase in PenH values. However, there should not be a group effect, meaning that both groups will have similar increases in PenH values as their respective concentration of methacholine increases.

To test the USV hypothesis, we ran a univariate ANOVA with the same two groups, but only used Pen H values from Dose 3 in the analysis. Only Dose 3 was used in order to maximize the amount of data available to us. Due to the humane method of our procedure that involved removing animals from the plethysmograph with significant signs of labored breathing and dangerously high PenH values, data was lost for some animals in the HDM-Meth group after

Dose 3. We ran one analysis with two factors: USV and group. We found no interaction between these two factors. We, therefore, re-ran the ANOVA with just the USV factor.

Chapter 3

Results

Prior to 36 days of age we were unable to reliably measure PenH (Figure 3-1), and after 42 days we had trouble keeping the plethysmograph functioning properly. Thus, the majority of the following analyses are based on PenH measures collected at PD 36.

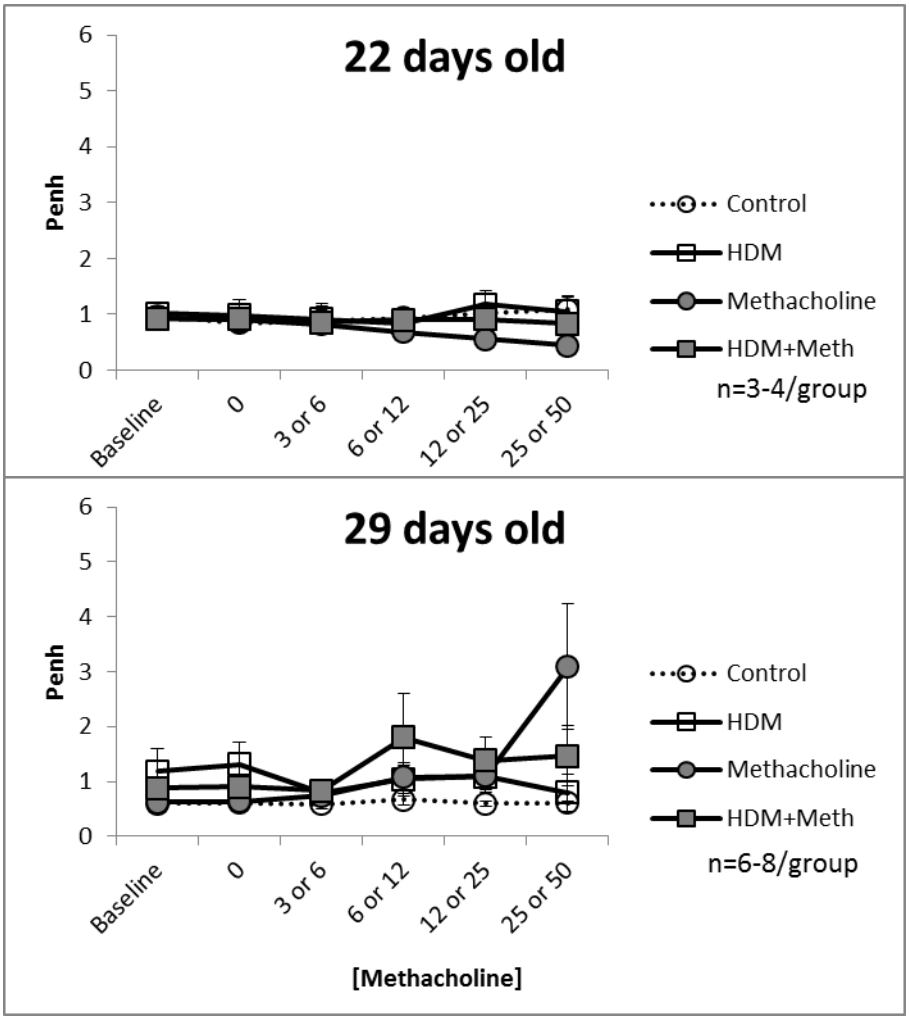

Figure 3-1: PenH of increasing methacholine dose for mice at PD 22 and 29. Each treatment group is represented by a different line and standard error bars are included.

Effect of Methacholine on Penh

Mice treated with methacholine had significantly greater PenH values than mice treated with saline ($F_{1,21}$= 16.06, p<.001), and methacholine dose had a significant influence on PenH values, with the highest dose leading to Penh values that were four times as great as the lowest dose of Penh (Time/dose * Methacholine interaction: $F_{5,105}$ = 5.66, p<.001; Figure 3-2).

Test of the dosage hypothesis

HDM-treated mice that received a ½ dose of methacholine had PenH values that did not differ from non-HDM-treated mice that received the full methacholine dose (Time/dose * HDM interaction: $F_{5,55}$=0.15, p>.95; Figure 3-2, Methacholine vs. HDM+Meth groups).

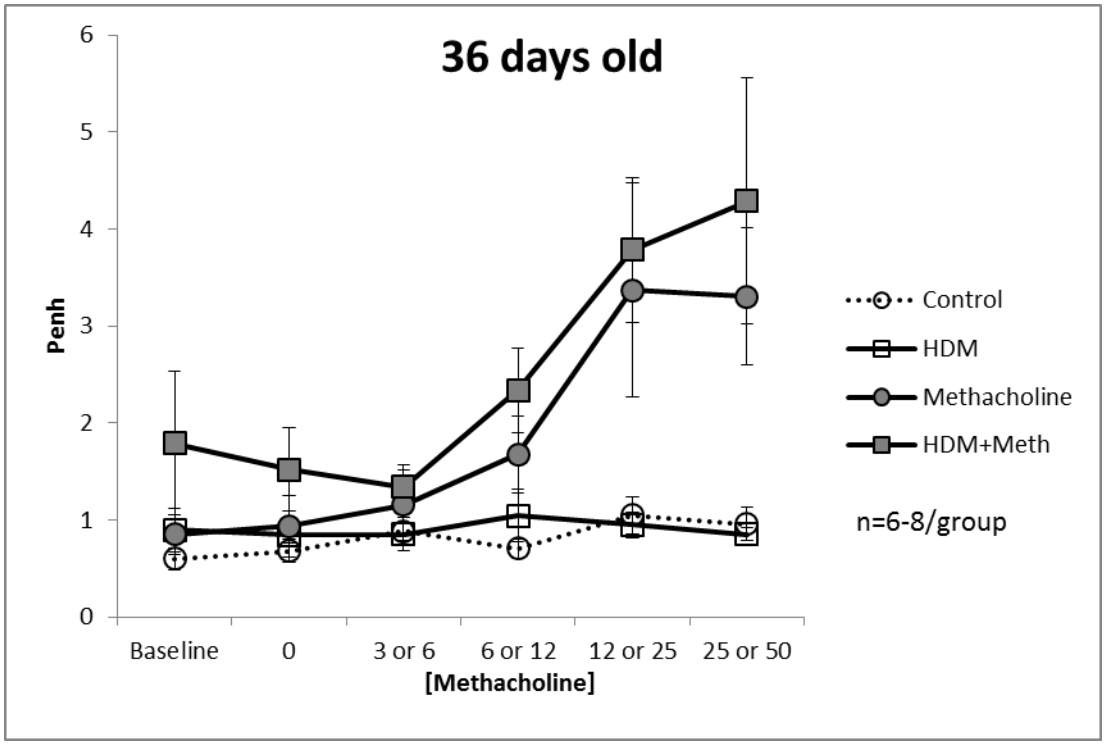

Figure 3-2: PenH of increasing methacholine dose for mice at PD 36. Each treatment group is represented by a different line and standard error bars are included.

Test of USV hypothesis

Mice that were characterized as High USV did not have greater PenH values in response to methacholine compared to Low USV mice ($F_{1,12}$=2.14, p=.17; Figure 3-3).

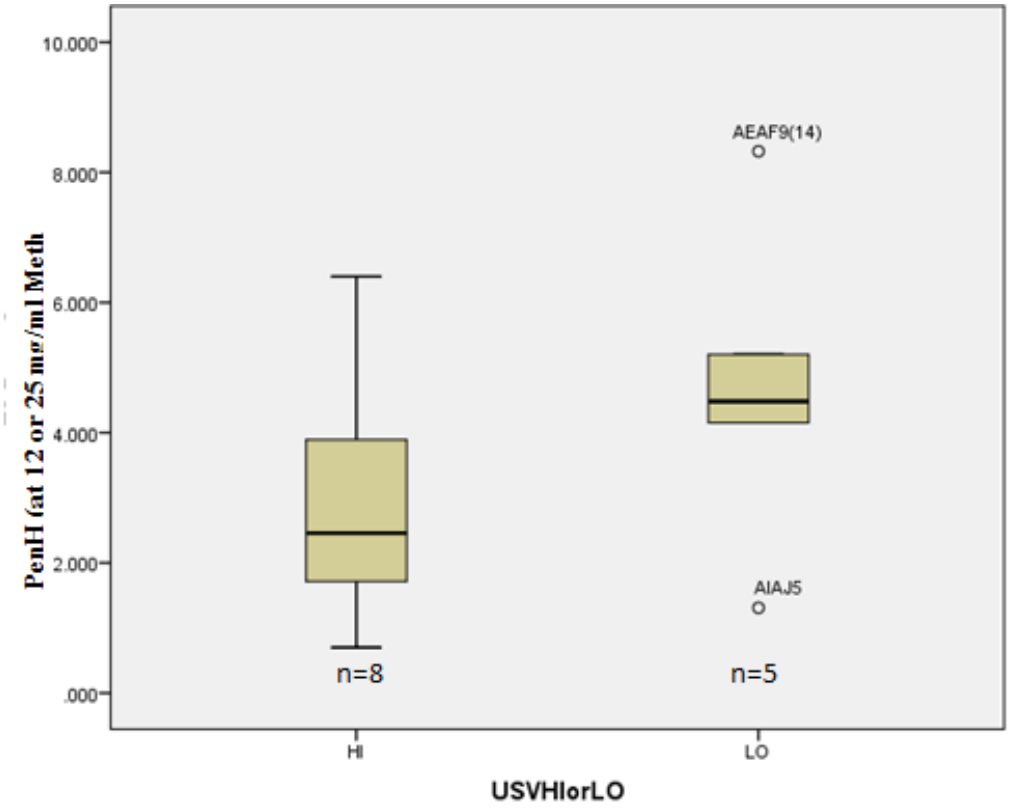

Figure 3-3: Box Plot of PenH values for (high and low) USV mice treated with methacholine. (Methacholine and HDM+Meth groups are combined). Black horizontal lines indicate means, top and bottom of yellow boxes indicate quartiles, bars indicate standard errors, and outliers are shown (AIAJ5 and AEAF9).

Chapter 4

Discussion

Accurate Dosage Compensation for Animals Treated with House Dust Mite Extract

The major finding of this study is that mice treated with HDM, a known allergen that causes airway inflammation, will show a similar lung response to a methacholine dose that is half of the dose given to mice that did not receive HDM. It was shown that this half dose of methacholine for the HDM exposed mice produced similar increases in PenH values compared to the mice that did not receive HDM. PenH values were significantly greater for the mice that received the methacholine treatment in comparison to those that received saline, demonstrating the significant effect of methacholine on lung function. Thus, the following hypothesis was supported: Mice pre-treated with HDM to cause lung inflammation only need a half dose of methacholine to cause lung function similar to a full dose in mice that are not pre-treated with HDM. This is important background information for the development of an animal model to test effects of asthmatic bronchoconstriction and lung function on mental health. These results allow us to mimic similar experiences of an asthma attack in both HDM and non HDM treated mice.

Establishing the proper methacholine dosage to treat mice that were exposed to HDM in comparison to mice that were not exposed to HDM is an important step for developing an animal model to study the effect of methacholine on lung function. Using methacholine to mimic an asthma attack in mice is a new practice. This method has been used to study lung function in humans with asthma, but using it to mimic an asthma attack in mice is new. It is especially important to this lab because we are attempting to show that methacholine is not only causing the experience of an asthma attack in periadolescent mice, but it is also inducing anxiety like

behavior. We modeled a previous study that successfully used HDM treatment to cause the allergic reaction in the airways of young mice. They found that their method of intranasal HDM administration (Appendix A) caused significant airway remodeling and airway hyperresponsiveness in mice that was comparable to symptoms reported with pediatric asthma (Saglani et al, 2009). Therefore, we used these findings to determine if this established method of induced inflammation in mice causes a significant difference in lung response to a known bronchoconstrictor, methacholine. In order to effectively determine this connection in the development of an animal model, it is essential to ensure that mice are receiving comparable doses of methacholine during the bronchial challenge test.

Buxco's whole body plethysmograph was used in this study to measure lung function during administration of methacholine. This machine allowed for the capturing and recording of PenH values, but it did have its drawback in terms of accuracy and reliability. Although we experienced technical difficulties with the machine throughout the duration of the study, there may be another reason that the plethysmograph was unable to detect and register PenH values at PD 22 and 29. It is possible that the lung size of periadolescent mice at this extremely young age is too small for the plethysmograph to register and detect changes in PenH values throughout methacholine treatment. The plethysmograph operates by sensing volume changes as a result of thoracic movement within the chamber. If lung size is too small, it is possible that the mice are responding to the methacholine treatment, but the plethysmograph is not sensitive enough to detect such minor changes in volume. A previous study showed a similar result using whole body plethysmography and PenH to analyze the effect of airborne allergens, specifically aerosolized ovalbumin, on the initiation of asthma in both neonatal and adult mice. For the neonatal mice in this study, PenH was measured between PD 16 and 28, a time period which our plethysmograph was unable to effectively record PenH. Their results showed that these neonatal mice displayed significant airway hyperresponsiveness after exposure to both the allergen and methacholine.

Despite showing significant hyperresponsiveness, in comparison to adult mice (56 days old), PenH values in the neonatal mice were three times lower at the highest dose of methacholine (Hamada et al., 2000).

It would make sense that younger mice would have greater difficulty with the same dose of methacholine as older mice due to their smaller lung capacity; however, the plethysmograph may not be sensitive enough to register such small changes in air volume within the chamber. In order to determine if HDM caused physiological changes in the lungs, Saglani et al, 2009 measured airway resistance and compliance in anesthetized mice and assessed airway hyperesponsiveness. This study showed that significant increases in airway hyperresponsiveness of mice treated with HDM occurred as early as PD 14, much earlier than we started seeing PenH increases in our study. However, Saglani et al, 2009 used a different measure of lung function: the Flexivent system. This system, although providing greater accuracy in detecting and measuring airway hyperresponsiveness to methacholine treatment, is much more invasive to the animal than whole body plethysmography. Therefore, this method would not be useful for our current study because putting our study animals through an invasive and stressful procedure in order to calculate airway hyperresponsiveness may have influenced post-treatment anxiety-like behavior. It is important to note that these results do teach us that Buxco's whole body plethysmograph may not be the optimal measure of airway hyperreresponsiveness; yet it does allow for the administration of methacholine in a noninvasive and controlled environment, making it useful to labs that are looking to model natural behaviors.

Early Life Anxiety and Lung Function

The second major finding of this study is that early life anxiety, measured by isolation-induced ultrasonic vocalizations (USV) in the first few days of life, does not play a significant

role in lung function during exposure to methacholine. Mice that demonstrated high anxiety early in life did not respond to methacholine with greater PenH values than those that demonstrated low anxiety. In fact, the results, although they were statistically insignificant, showed that the opposite may be true. Mice that demonstrated low anxiety early in life actually showed greater PenH values in response to the same dose of methacholine as the high anxiety group. Thus, the following hypothesis was not supported: Mice that have high anxiety, measured by isolation-induced USV, will respond with greater PenH values when exposed to the same dose of methacholine as the low anxiety group.

Previous research in the human population has shown that high anxiety creates increased symptom perception during bronchoconstriction (Spinhoven et al., 1997), so it was expected that high anxiety mice would respond to the same dose of methacholine with higher PenH values than low anxiety mice. In the prior human study, baseline anxiety for participants was determined by combining results of the Borg scale, which accounts for perceived breathlessness, and the subjective unit of distress scale (SUDS). The anxious perceivers had significantly higher perceived breathlessness and anxiety during the bronchial challenge test. In addition, the anxious individuals were more accurate in their perception of airway obstruction (Spinhoven et al., 1997). This finding allowed researchers to conclude that anxiety before and during bronchoconstriction may result from increased sense of airway obstruction and pulmonary distress. Therefore, we expected that the high anxiety mice in this study would be significantly more reactive to methacholine administration due to their heightened sense of airway obstruction.

Another study of children supports the findings that anxiety is associated with heightened perception of symptoms during bronchoconstriction; however the researchers found an important timing component to this connection (Chen et al., 2006). They discovered that anxiety was only associated with heightened perception of symptoms when the child's asthma was mild, but not after administration of the methacholine challenge. Their findings suggest that anxiety only plays

a role in lung function when symptoms are mild, but our study compared anxiety to lung function at a high dosage of methacholine in order to attempt to find a connection between the two. For future studies, it may be more useful to evaluate the connection between anxiety and baseline levels of PenH.

Although this result was unexpected, it is important to try to understand why high anxiety mice did not show an increased lung response in comparison to low anxiety mice when treated with methacholine. First and foremost, it is important to understand that our initial hypothesis was based on human findings, which measure perception of symptoms during an asthma attack. This psychological measure is different than the physiological measure of PenH that we recorded in our mouse model, which might explain the unexpected results.

Although the results were not statistically significant, the trend was that the low anxiety mice actually responded with greater PenH values during exposure to the same dose of methacholine as the high anxiety mice, so it is important to address why this may have been the case. It is possible that mice that exhibited low anxiety during the USV collection period showed greater lung sensitivity during administration of methacholine because they were more surprised by the induced bronchoconstriction due to their usual low anxiety. Mice that show low levels of postnatal anxiety may not be used to experiencing frequent stress, so they may react more severely to a stressful situation, such as methacholine induced bronchoconstriction. However, mice that exhibit high postnatal anxiety may be used to being in a constant state of stress. If this is the case, they may not react as negatively towards stressful situations as the group that is not used to stress. An alternative hypothesis to explain this unexpected result could be that the mice that were high postnatal anxiety mice were not registering high USV call rates because their lungs were more sensitive; therefore, making it painful to call out and produce USVs. If this was the case, then the experimental groups would not have been accurate and could explain the unexpected result that low USV callers seemed to respond to methacholine treatment with higher

PenH values. These are two alternative hypotheses that could be considered for future research on the possible connection between anxiety and lung function in both the human population and in an animal model. This connection is especially important in the human population because if it is found that high anxiety in children can exacerbate symptoms of asthma, it is vital to recognize and treat anxiety disorders early in life.

Limitations and Future Considerations

We experienced both minor and major problems during this study that limited the quantity and quality of the results that were obtained. The major problem involved technical difficulties in both operating the plethysmograph and collecting PenH values from the plethysmograph. We began the study with 81 animals, all who received intranasal HDM/saline administration then proceeded to receive methacholine/saline treatment in the plethysmograph. However, data were only recorded for 30 of these animals. This occurred because the plethysmograph was not functioning properly and completely failed to record data following PD 42. Therefore, the statistical power of this study was severely weakened because not every animal could be included in the data analysis.

In addition, as seen in the HDM-Meth and Meth groups in Figure 3-2, the PenH values registered by the plethsymograph produced inconsistent and noisy data. As mentioned previously, there is strong criticism surrounding the use of PenH as a measure of airway hyperresponsiveness (Lunblad et al., 2007). In addition, there is no current way to establish a meaningful scale for PenH values, which may have produced the noise that was seen in Figure 3-2. For example, a doubling in PenH does not necessarily correspond to a doubling in labored breathing. This makes it difficult to quantify and compare PenH values between two groups, especially when the PenH values are close in value, as seen in Figure 3-2 between the HDM-Meth and Meth groups.

Fortunately for this study, observing that these two groups had similar PenH values, regardless of the lack of a valid numerical scale, was enough to establish that the dosage compensation was correct.

For this study and future studies, it is also important to acknowledge the potential weaknesses of USV analysis as an indicator of anxiety. As mentioned previously, some argue that USVs are more of a thermoregulatory process than a true indicator of anxiety (Blumberg et al., 2001). The counter argument to this is that anti-anxiety drugs have been shown to reduce USV output in young mice, which would suggest that USVs are a result of increased anxiety in young mice (Takahashi et al., 2009). However, there is still debate on this issue because the active doses of anti-anxiety drugs given to small pups could be close to producing sedation and muscle relaxation, which would result in fewer vocalizations. If this is the case, the reduced USV output would be more of a result of a drowsy state than a decreased sense of anxiety (Sanchez, C. 2003). USV as a measure of anxiety has been used in many previous studies because it is a noninvasive technique to easily gather information on initial anxiety-like behavior, but it is important to recognize for future studies that this measure can be highly variable and should be used more as an estimate than a definitive indicator of anxiety.

In moving forward from this study, it is important to understand the broad implications of our findings. First and foremost, we found that it is possible to administer a comparable dose of methacholine to mice that that have been exposed to an allergen that causes airway inflammation and to mice that have not been exposed to this allergen. This is important in the development of an animal model for asthma because it is necessary to find the correct dose of methacholine to administer to both treatment groups in order to induce a similar lung response. Also, we have shown that it is important to remember that both PenH and USV measures can be highly variable in an animal model. Overall, both of these findings can be beneficial to future developments in the animal model for asthma.

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Appendix A

HDM and Methacholine Treatment Protocols

Written by: Sonia A. Cavigelli

Last Updated: April 4, 2011

Intranasal HDM Administration

On Tuesday (01 June 2010), we visited Avery August's lab to learn the intranasal administration technique. This method was be used to administer house dust mite extract (allergen) to mice in the 'PSIN Asthma-Anxiety' study. We worked with Fei Huang (post-doc) & Arun Kumar (2nd year grad student). Present from our lab: Sonia Cavigelli, Kerry Michael, Danielle Cardell, Sumi Gnanarajah, Hashim Chaudhry. Neonatal House Dust Mite extract administration protocol used in PSIN Asthma-Anxiety study was taken from Saglani et al. (2009).

Supplies/Equipment Needed:

- Properly diluted allergen solution – there are 100 µl aliquots of correctly diluted solution in small tubes in CBL Rm 188 freezer. Remove/thaw one vial from freezer for each 5-6 mice that you need to treat.
- Sterile saline – also in 100 µl aliquots in freezer
- Pipettor and pipette tips (10 or 100 µl tips)

- Clean (disinfected) container to hold mouse that can be attached to anesthesia machine (e.g. glass 1-litter flask)
- Paper towels
- Gloves
- Isofluorane
- Code sheet indicating which mice to treat with allergen and which to treat with saline

Procedure:

1) For all mice older than 3 weeks of age, set up anesthesia machine. Make sure there is enough isofluorane in tank. Place a clean container (i.e. uninfected) from our lab onto the anesthesia machine vent table, place a paper towel in container, put the output tube of anesthesia machine into container, and cover with a lid. Turn anesthesia machine on and 'fill' container with isofluorane fumes. (For mice less than 3 weeks, there is no need for isofluorane.)
2) Place mouse into container and watch until its heartbeat has reduced to 1 beat/second. During this time, you can load your pipette tip with the proper volume of either allergen solution or saline – consult code sheet. Use a new pipette tip for each mouse. Volume for mice less than 3 weeks = 10µl, volume for mice older than 3 weeks = 15µl. At the appropriate time, remove mouse from container and hold it on its back in your non-dominant hand, pinching the scruff of the neck between thumb and forefinger and allowing its body to lie horizontal in your hand.
3) With dominant hand, hold pipettor at a 30 degree angle from horizontal and release a little of the allergen solution so that it forms a drop on the outer edge ofthe pipette tip. Touch the drop of solution to the mouse's nares and allow them to breathe it in. Keep

working like this until the full volume of allergen has been administered. Be careful to allow the mouse to take a breath between each drop administered, but work quickly so that you do not need to give the mouse another dose of isofluorane.

4) Once the full dose has been administered, return mouse to home cage and observe it to make sure it recovers from anesthesia. Return to cage rack after full recovery.

Methacholine Administration

Setting Up an Experiment

NOTE: You may skip this part of the procedure by loading a pre-existing setup. Select File>Open>“Mouse Asthma and Anxiety”

1) Right-click on Experiment node and choose Add> Site
2) Right-click on Site node and choose Add> WBP (for whole-body plethysmograph)
3) Right click on WBP>setup to set up analyzer
    a. Logging type should be “Event Based”
    b. On Input tab, set Connection to Lead 1
4) Right-click on WBP1>Show Views
    a. Select Add> Strip Chart
        i. Right-click on table and select Select Table
        ii. Choose the bottom-most icon (WBP1)
    b. Select Add> Fast Graph
        i. Right-click on graph and select Add Trace
        ii. Click on Outputs
        iii. Select Penh from that list
5) Right-click Site 1 and rename to Mouse 1

6) The whole previous bit sets up for one mouse. To copy all of this to have one site for each mouse:
    a. right-click on WBP1>Copy Items and select Analysis, Views, and Settings
    b. Right-click Experiment>Add> Site
    c. Right-click Site> Paste WBP

Calibration

1) Set silver switch on pre-amp to OFF
2) Click System>Calibrate
3) Pull 1mL of air into the attached syringe
4) Turn the zero knob until the signal is at zero (for large adjustments, use the Balance screw with the tiny screwdriver)
5) Flip the silver switch to DC and re-zero
6) Push 1mL of air from the syringe into the chamber and hit F7
    a. Accept the settings the computer gives you
7) Flip the silver switch to AC
8) Save and close
9) Open “Asthma and Anxiety Study”

Loading the Animal

1) Holding the mouse by the tail, place the front end of it on the grate in the chamber. Pull gently to make the mouse hold on to the grate.
2) Let the mouse go – it will circle inside the chamber and pull its tail in behind it.
3) Close the chamber. Make sure the THING on top is open (parallel to tubing)

Collecting Data

1) Click Run button (little man running)
    a. Select the Mouse Asthma and Anxiety study
    b. Under Session Description, enter which litter you are running and the date
    c. Enter the appropriate mouse IDs under “subject ID” for each site. Make sure to note down which mouse ID is at which site on a separate paper or document for later reference.
    d. Click Go!
2) Select Mouse1>Protocol on the Command Menu, the box on the left side of the screen. Below the Command Menu, a box displaying the protocol task list should be observed along with several command options (i.e. ‘Go On’, ‘Restart’, ‘Stop’). Protocol Status should be displayed atop the task list, at this point counting down from 3 minutes for the mouse’s acclimation period.
    a. If the protocol task list is not displayed, select the Mouse1 tab on the lower box to display it. Data will be automatically collected after the acclimation period ends.
    b. When the protocol reaches task 9, add PBS to the nebulizer and click the ‘Go On’ Command below the task list. You are given 10 minutes for this step, before the protocol will continue on its own.
    c. When the protocol reaches task 14, add Methacholine (or saline for controls) to the nebulizer and click the ‘Go On’ Command below the task list. You are given 10 minutes for this step, before the protocol will continue on its own.
    d. When the protocol reaches task 18, data collection for the first mouse ID is complete. Move on to Step 3.

3) Load the next animal and select Mouse2>Protocol to view the protocol task list for the next mouse ID. The protocol should be paused, counting down from a time in the 900 minutes range.
    a. If the task list is not displayed, see step 2a.
    b. Select ‘Go On’ to begin the 3 minute acclimation period and following data collection task.
    c. On task 10, add PBS and click ‘Go On’.
    d. On task 15, add Methacholine (or saline for controls) and click ‘Go On’.
    e. At task 19, data collection for the second mouse ID is complete. Move on to Step 4.
4) Follow Step 3 for the remaining animals, with appropriate protocols selected per trial (Mouse3>Protocol, and so on) until all the animals have undergone the procedure.

Click the Stop button (To the right of the Run button).

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Education

B.S., Biology, May 2013, The Pennsylvania State University, University Park, PA

Honors and Awards

- President's Award, The Pennsylvania State University: April 2010
- Dean's List, The Pennsylvania State University: Fall 2009-Spring 2013
- Schreyer Honors College Scholarship Recipient: Fall 2009-Spring 2013

Association Memberships/Activities

- Phi Beta Kappa
- Phi Kappa Phi

Professional Experience

- Behavioral Neuroendocrinology Lab, The Pennsylvania State University
  Undergraduate Research Assistant, Spring 2011 to Spring 2013
  - Operate the plethysmograph for administration of methacholine treatments
  - Assist in coding behavioral data
  - Administer treatments to and participate in the handling of mice
  - Participate in weekly lab meetings where various research articles are presented and discussed
- The Reading Hospital and Medical Center
  Surgical Care Assistant, Summer 2011 and Summer 2012
  - Assisted doctors and nurses with prepping patients for surgery
  - Gathered supplies for cases
  - Transferred patients from operating room to recovery

Research Interests

I have a broad interest in the connection between psychological factors of human health and physiological factors. Specifically, I am interested in the role that anxiety plays in the life of an asthmatic individual during an acute asthma attack. I am also interested in researching the possible connection between early life anxiety and asthma later in life.