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Peri-adolescent asthma symptoms cause adult anxiety-related behavior and neurobiological processes in mice

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Abstract

Human and animal studies have shown that physical challenges and stressors during adolescence can have significant influences on behavioral and neurobiological development associated with internalizing disorders such as anxiety and depression. Given the prevalence of asthma during adolescence and increased rates of internalizing disorders in humans with asthma, we used a mouse model to test if and which symptoms of adolescent allergic asthma (airway inflammation or labored breathing) cause adult anxiety- and depression-related behavior and brain function. To mimic symptoms of allergic asthma in young BALB/cJ mice (postnatal days [P] 7–57; N=98), we induced lung inflammation with repeated intranasal administration of house dust mite extract (most common aeroallergen for humans) and bronchoconstriction with aerosolized methacholine (non-selective muscarinic receptor agonist). Three experimental groups, in addition to a control group, included: (1) “Airway inflammation only”, allergen exposure 3 times/week, (2) “Labored

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breathing only”, methacholine exposure once/week, and (3) “Airway inflammation + Labored breathing”, allergen and methacholine exposure. Compared to controls, mice that experienced methacholine-induced labored breathing during adolescence displayed a ~20% decrease in time on open arms of the elevated plus maze in early adulthood (P60), a ~30% decrease in brainstem serotonin transporter (*SERT*) mRNA expression and a ~50% increase in hippocampal serotonin receptor 1a (*5Htr1a*) and corticotropin releasing hormone receptor 1 (*Crhr1*) expression in adulthood (P75). This is the first evidence that experimentally-induced clinical symptoms of adolescent asthma alter adult anxiety-related behavior and brain function several weeks after completion of asthma manipulations.

Keywords

Adolescence; Anxiety; Asthma; House dust mite; Methacholine; Sex Differences

1. INTRODUCTION

Adolescence is a unique and critical period of behavioral and neurobiological development with rapid and substantial maturation of brain regions and neurotransmitter systems involved in emotion regulation [1–7]. Repeated exposure to health and environmental challenges during this stage of life alter behavioral and physiological development related to internalizing disorders like anxiety and depression [8–10]. Allergic asthma represents a common chronic health challenge, affecting 25.7 million people (9.5% <18y/o, 7.7% >18y/o) in the United States [11], and it is associated with internalizing disorder comorbidity occurring as early as adolescence [12–17]. Asthma symptoms are significant chronic stressors, and asthma is associated with glucocorticoid (GC) dysregulation and exogenous corticosteroid administration as medication [18–21]. Altered GC exposure in asthma, particularly during adolescence, may be a mechanism that primes individuals for internalizing disorders [22–28].

GCs affect neurons at the functional and transcriptional levels [28–30]. Human studies indicate a strong relationship between peri-adolescent stress and adult GC regulation and mental health [10,24]. Adults that experienced peri-adolescent abuse have lower basal cortisol levels and dampened cortisol responses, and GC dysregulation is a risk factor for anxiety and depression [24,26,28,31–34]. Adolescents may be particularly vulnerable to GC influences on hippocampal gene regulation and down-stream GC dysregulation [22,24,35–38]. Rodent models confirm that pre- and peri-adolescent chronic stress can have long-term influences on GC regulation and anxiety- and depression-related behaviors [39,40]. For example, chronic restraint or social stress elicit GC receptor (GR) downregulation in the prefrontal cortex (PFC), a flattened GC circadian rhythm, and increased anxiety- and depression-like behavior [33,40–42].

Allergic asthma has two distinct components that may affect GC production and/or anxiety/depression development. First, allergic asthma involves increased airway inflammation, which is associated with increased cytokine production that can alter GC production, potentially increasing risk for internalizing disorders [20,43–45]. Allergic asthma is driven

by a predominant T helper 2 (Th2) response, and Th2 cytokines, interleukin 4 (IL-4), interleukin 5 (IL-5), and interleukin 13 (IL-13), are critical for the development of this disease. In various animal models and in humans, antibodies that block these cytokines have had varying efficacy in treating the symptoms of asthma [46–48]. Second, asthma involves unpredictable respiratory distress with heavily labored breathing. This hypoxic state can lead to limited airflow, increased airway muscle activity, risk for respiratory failure, and airway remodeling [49,50]. Given these two distinct symptoms of asthma – lung inflammation and labored breathing – we independently manipulated these two features in a mouse model.

To understand mechanistic links between peri-adolescent asthma and adult internalizing disorders, we validated a rodent model by experimentally testing if lung inflammation and/or labored breathing during peri-adolescence led to significant adult symptoms associated with human internalizing disorders. We quantified anxiety- and depression-related symptoms in adulthood by measuring exploratory and hedonic behavior, basal GC regulation by measuring serum and fecal GC concentrations, GR and mineralocorticoid receptor (MR) gene expression in the hippocampus [24,26,28,34,42], and serotonergic and corticotropin-releasing hormone (CRH) receptor/transporter gene expression in brainstem, hippocampus, and PFC [51–57]. Because sex differences exist in susceptibility to asthma and internalizing disorders, we conducted the study with both male and female mice [10,58–63].

2. MATERIALS AND METHODS

A longitudinal study was used to induce peri-adolescent allergic asthma symptoms and measure adult behavioral and neurobiological outcomes related to internalizing disorders. BALB/cJ mice – a strain susceptible to allergic airway inflammation and hyperresponsiveness [64,65] – were used. Four peri-adolescent conditions included: Airway Inflammation (AI), Labored Breathing (LB), Airway Inflammation + Labored Breathing (AI +LB), and Similarly-Handled Controls (CON; Figure 1A). Three consecutive cohorts were bred, providing 9+ offspring of each sex per condition (N=98 mice, 30–38 mice/cohort). Litter effects were controlled by evenly distributing same-sex siblings across all four conditions (3 experimental and 1 control). Each experimental group consisted of male and female pups selected from 14 litters (each experimental/control group had 12–14 males and 9–12 females). All procedures followed the National Institute of Health guide and were approved by the Pennsylvania State University IACUC committee.

2.1 MOUSE BREEDING AND HOUSING

BALB/cJ breeders were obtained from Jackson Laboratories (Bar Harbor, ME). Sister-pairs were mated with one male to produce ‘double-litters’ (14 double-litters, mean size: 9.7 pups, not culled). Pups were marked daily with non-toxic Sharpie ® marker for identification until given a unique ear notch (P9). To control for pup differences in pre-manipulation anxiety-prone phenotypes, we measured ultrasonic vocalizations (USV) on P3-5 (2 minutes/day) using the ‘Isolation’ method and recording at 65 Hz [66–69]. Consistently high- or low-calling pups within each litter were selected and equally-distributed among conditions. Pups were weaned at P22 into same-sex sibling groups in standard cages with corn-cob bedding

(2–4 pups/cage, 28 cm x 17 cm x 12 cm). At P50, mice were individually housed in standard cages containing a red polypropylene tube for environmental enrichment and for low-stress transportation out of the home cage [70,71]. Colony and testing rooms were maintained at $21\pm 1^{\circ}\text{C}$ on a reverse 12:12 light:dark schedule (lights on 18:00 hours, lights off 06:00 hours) with *ad libitum* access to food and water. Labored breathing, fecal corticosterone metabolites, and anxiety-related behavior were measured from all offspring (n=98; 54 male, 44 female; 9–14 pups/sex/condition). Depression-related behavior was measured in half of these offspring (n=53; 30 male, 23 female, 5–9 pups/sex/condition); this smaller sample size was a result of procedural error for half of the mice. Lung mucus/inflammation/collagen, brain gene expression, and basal circulating corticosterone were measured in a subset of the 98 offspring (n=54; 28 male, 26 female; 6–8 pups/sex/condition); lung cytokine and circulating cytokine levels were measured in a third of the offspring (n=38; 19 male, 19 female; 4–5 pups/sex/condition).

2.2 INDUCTION OF PERI-ADOLESCENT ALLERGIC ASTHMA SYMPTOMS

2.2.1 Airway Inflammation—Young mice in the AI and AI+LB conditions were exposed to extract of *Dermatophagoides pteronyssinus*, commonly referred to as house dust mite (HDM; Greer Labs, NC) to induce chronic lung inflammation (3x/week; Figure 1B). Ovalbumin, often used to induce lung inflammation, was not used because tolerance occurs with repeat exposure [72,73]. During P7-15, mice received 10 μg HDM (10 μL of 1mg/ml protein weight solution in saline) per exposure. During P16-56, the dose increased to 15 μg HDM (15 μL) and was given under brief isoflurane anesthesia [65]. This method produces significant lung inflammation within 2 weeks of first dosage, which persists throughout treatment [74]. LB and CON mice were exposed to a control treatment of intranasal saline using the same exposure schedule, volumes, and procedures as those for HDM administration, including repeated exposure to isoflurane to administer the saline (Figure 1A).

2.2.2 Labored breathing (LB)—Young mice in the LB and AI+LB conditions were exposed to an inhaled muscarinic receptor agonist, methacholine (MCH; Sigma, St. Louis, MO), once per week during P22-57 (Figure 1B). To measure airway constriction/labored breathing, a whole-body plethysmograph was used (Data Sciences International, New Brighton, MN) for repeated non-invasive measures. Mice were placed in the plethysmograph chamber (diameter 7.5cm, height 7cm) and allowed to acclimate for 3 minutes, then exposed to five increasing doses of aerosolized MCH for 3 minutes per dose [65]. To induce similar levels of labored breathing in LB and AI+LB conditions, AI+LB mice received half-doses relative to LB mice (LB vs. AI+LB MCH doses: 0, 6.25, 12.5, 25, and 50ng/mL vs 0, 3.13, 6.25, 12.5, 25ng/mL in 100 μL saline) based on preliminary data and prior studies [65]. AI and CON mice received the control treatment of aerosolized phosphate-buffered saline (PBS) in the plethysmograph chamber on the same time course used for MCH administration as explained above (Figure 1A). To verify MCH-induced bronchoconstriction for each dose, we recorded labored breathing behavior as present or absent, and we recorded and calculated mean enhanced pause (Penh) [75] with FinePointe software.

2.3 BEHAVIORAL TESTING

2.3.1 Anxiety-Like Behavior, Elevated Plus Maze (EPM)—At P60, anxiety-like behavior was measured on EPM, a common, pharmacologically-validated test for anxiety in mice [76–80]. Two open (30cm x 5cm) and two closed (30cm x 14.5cm x 5cm) flat perpendicular arms were elevated 42cm above the ground. Test orders were pseudo-randomized to balance across conditions. Mice were transported to the test room in their home cages approximately 1 hour before testing. Testing was conducted during the latter half of the dark phase (13:00–17:00 hours) under red-light illumination (<5 lux). Mice were placed in the center of the arena facing an open arm and video-recorded for 5 minutes. Entry into the maze arms was defined as 2-limbs passing the threshold boundary. EPM videos were scored for percent time on open arms, proportion of entries into open arms, and total number of entries into open and closed arms. Decreased time on or entries into open arms were used to quantify anxiety-like behaviour; arm entries were used to quantify locomotion [80].

2.3.2 Hedonic Behavior, Sucrose Preference Test (SPT)—On P66, we assessed anhedonic behavior by measuring free-choice sucrose consumption in SPT [81]. Forced swim test and tail suspension test were not used because performance in these tests (swimming and righting) are dependent on peak lung function, which was compromised in AI and AI+LB mice, regardless of affective state [82]. Mice were given 24 hours of free access to two water bottles, one with tap water and the other with 3% sucrose solution [83]. Bottle positions were switched after 12 hours to avoid side preference biases. Bottles were weighed before and after the 24-hr test to measure sucrose solution consumption relative to water. Decreased preference for sucrose solution is a sign of anhedonic behavior [3].

2.4 PHYSIOLOGICAL OUTCOMES

2.4.1 Corticosteroids in Feces and Serum—The immediate effects of peri-adolescent asthma symptoms on basal GC production were measured by collecting feces on P59 (late adolescence). Corticosteroids were extracted and measured from feces to provide a non-invasive measure of the circadian rhythm. These measures involved the following procedure and analysis for every experimental group (CON, AI, LB, and AI+LB). On P50, mice were moved from group-housing, solid-bottom cages to individual-housing, wire-bottom cages, which provided an 8-day acclimation period prior to fecal collection. Previous research has demonstrated that individual housing is a stressor for rodents [3,40,84], thus all experimental and control animals experienced the same individual housing. On P59, samples were collected every 4 hours, with samples at 08:00 and 12:00 hr (i.e. 2 and 6 hours after lights off) providing an estimate of daily peak production, and samples at 20:00 and 0:00 hr (i.e. 2 and 6 hours after lights on) providing an estimate of daily trough (accounting for lag time for circulating steroids to be metabolized and excreted in feces) [85–89]. All animals remained in individual housing in solid-bottom cages for the remainder of the study. To measure longer-term effects of peri-adolescent asthma on GC production, mice were sacrificed three weeks after asthma treatments (~P76; adulthood). Heart blood was collected on average 6 minutes from initial cage disruption and stored on ice until serum separated and stored at –80°C. Commercial [¹²⁵I] radioimmunoassay kits (MP Biomedicals, Solon OH) were used to measure corticosteroid concentration in fecal extracts and serum using

published methods [85–87]. Time to collect blood did not relate to serum corticosterone concentration (Pearson $r=0.037$, $p=0.40$, $n=54$), likely because mice were habituated to cage disturbance, which was a regular occurrence throughout the study, and mice were rapidly sacrificed and blood collected once their cage was disturbed. Samples were analyzed in duplicate; intra- and inter-assay coefficients of variation for low and high controls were 16.04 and 5.27, and 24.83 and 16.62 respectively.

2.4.2 Lung Mucus, Inflammation, and Collagen—Lung sections were preserved in formalin and paraffin-embedded, sliced, and stained to visualize mucus (periodic acid-Schiff stain, PAS), inflammation (hematoxylin and eosin stain, H&E), and collagen (Masson's trichrome stain, TRI). For each stain, 3 consecutive slices were mounted from posterior portions of the right inferior and left lobes. Mucus levels were quantified on a scale of 0–6 with increasing numbers indicating increasing mucus (Table 1). All 6 slices were quantified and an average mucus score calculated per mouse. Inflammation patches were counted if adjacent to blood vessels or airways, and thickness of the three largest patches were measured perpendicular to the airway/vessel membrane (20 μ m diameter or larger). In statistical analyses, we used total patch number and mean patch thickness calculated from the six largest patches (three from each lung)/mouse. If fewer than three patches were present, we quantified and averaged as many patches as possible. Collagen thickness was quantified around airways at least 150 μ m diameter, with five thickness measures for each of 3–5 airways on each of two slices per mouse. Mean collagen thicknesses across all airways was calculated for each mouse.

2.4.3 Adult Lung Cytokine Gene Expression—At sacrifice, lungs were freshly dissected and stored in RNAlater (Ambion, Carlsbad, CA) for 24 hours prior to freezing at -80°C . Total cellular RNA was extracted from tissue using TRIzol reagent (Invitrogen; Carlsbad, CA) and Qiagen RNeasy columns (Qiagen; Germantown, MD). RNA quantity was measured with a NanoDropTM spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and RNA quality checked with an Agilent 2100 BioAnalyzerTM (Agilent Technologies, Santa Clara, CA). RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription kits (Applied Biosystems; Wilmington, DE). Resulting complementary DNA (cDNA) was examined for relative abundance of *IL-4*, *IL-5*, and *IL-13* (Life Technologies, Mm00445259_m1, Mm00439646_m1, Mm00434204_m1) using quantitative real-time PCR (qRT-PCR). qRT-PCR reactions were prepared in triplicate in 96-well plates with validated TaqMan probes and the constitutively expressed beta actin gene (*Actb*, Life Technologies, Mm02619580_g1). The following cycle settings were used on the StepOnePlus Real-Time PCR System (Applied Biosystems, Wilmington, DE): 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C test for 60 seconds. Target gene expression was normalized to *Actb* expression by subtracting each sample's gene of interest's cycle threshold (CT) values from the corresponding *Actb* CT value, generating ΔCT values. Difference scores were standardized to the median CON mouse value, with all expression levels given relative to this median value. Relative abundance of each transcript per sample was determined using the $2^{-\Delta\text{CT}}$ method.

2.4.4 Adult Circulating Cytokine Levels—Serum was collected from whole blood obtained at sacrifice via mouse cardiac puncture (centrifugation at 12,000x g, 5 minutes, 4°C) and stored at –80°C. Cytokine concentrations (IL-4, IL-5, IL-6, IL-13, TNF α) were measured with MILLIPLEX® MAP Multiplex Immunoassay Kits (Mouse Cytokine/Chemokine Panel I, MCYTOMAG-70 K; Merck Millipore; Darmstadt, Germany). A Bio-Plex 200 System (Bio Rad; Hercules, CA) using a five-parameter logistic regression model created standards curves and calculated mean sample concentrations.

2.4.5 Adult Brain Serotonin- and HPA-Related Gene Expression—At sacrifice, brains were freshly-dissected to separate brainstem, hippocampus, and PFC. All sections were collected, processed, and analyzed as previously described for lung cytokine expression. Brain tissue TaqMan Gene Expression Assay primers and probes used included: *SERT*, *5Ht1ra*, *Crhr1*, GR (*Nr3c1*), and MR (*Nr3c2*; Life Technologies, Mm00439391_m1, Mm00434106_s1, Mm00432670_m1, Mm00433832_m1, Mm01241596_m1).

2.5 STATISTICAL ANALYSES

Repeated measures ANOVAs were used to compare Penh across conditions. One ANOVA was conducted for each administration age (P22, 29, 36, 43, 50, 57) and the repeated measure at each age was mean Penh during each MCH dose. To compare behavioral and physiological outcome variables among conditions, all ANCOVAs were run with the following factors (each with 2-levels) – AI (intranasal saline vs. HDM exposure), LB (inhaled aerosolized saline vs. MCH exposure), sex (male vs. female), and USV category (high vs. low) – and with a covariate of mean cohort outcome value. The cohort covariate was used because *a priori* analyses (ANOVAs) indicated that several outcome variables were affected by cohort. All main and interaction effects with $p < 0.05$ were reported. When ANCOVAs identified sex interactions, males and females analyses were conducted separately to determine specific sex effects. We compared results between each of the four experimental groups (CON, AI, LB, AI+LB), using ANCOVAs as described above with the AI and LB factors replaced with a 4-level Group factor. Outcomes were compared among the four groups using contrasts to compare the three experimental groups to the control group. Further, non-hypothesis-driven pairwise comparisons were conducted among the four groups using Sidak correction for multiple comparisons. Results of these post-hoc analyses are reported in the figure legends. For all tests, variable distribution was checked for normality; several variables (mucus, fecal corticosterone, serum corticosterone and cytokines, and gene expression) were log-transformed to achieve normal distribution for parametric statistics. No outliers were identified. As a result of technical issues, there was one missing data point in each of the following outcome variables: elevated plus maze time on open arms, circulating IL-4 concentration, and lung mucus, inflammation, and collagen measures. All other outcome variables included the full sample size indicated in Section 2.1. Figures depict untransformed estimated marginal means.

3. RESULTS

3.1 PERI-ADOLESCENT BRONCHOCONSTRICTION (P22-57)

MCH led to increased labored breathing at every age of administration (Figure 2; Table 2). Penh values were not reliably higher in LB vs non-LB mice at P22 and 29, but were significantly higher at P36, 43, 50, and 57 (Figure 2; Table 2).

3.2 LATE ADOLESCENT FECAL CORTICOID RHYTHM (P59)

Fecal peak and trough corticoid concentrations were 6 times higher in females than males (Sex effect for peak and trough: $F_{1,81}=209.50$, $p<0.0001$ and $F_{1,81}=268.63$, $p<0.0001$). LB females trended towards elevated trough corticoid levels relative to non-LB females, whereas males showed an opposite trend (ANCOVA, Sex x LB interaction: $F_{1,81}=5.68$, $p<0.05$; Figure 3). There were no effects of AI or USV, nor any interaction effects, on trough fecal corticoid production ($F_s<2.44$, $p_s>0.12$). Peak fecal corticoid production was not affected by AI, LB, USV, or any interactions ($F_s<3.60$, $p_s>0.10$).

3.3 ADULT BEHAVIOR

3.3.1 Elevated Plus Maze (P60)—Peri-adolescent LB caused increased anxiety-related behavior in young adults. LB animals spent less time on the open arms ($F_{1,80}=5.230$, $p<0.05$; Figure 4A) and had a lower proportion of open arm entries than non-LB mice ($F_{1,81}=4.220$, $p<0.05$; Figure 4B). There were no significant differences in locomotion (number of entries into open and closed arms) among LB groups ($F_s<1.804$, $p_s>0.183$; Figure 4C). There were no main effects of sex, AI, or USV, and no significant interaction effects for any of the EPM behaviors ($F_s<2.195$, $p_s>0.142$).

3.3.2 Sucrose Preference Test (P66)—Neither AI nor LB predicted the relative amount of sucrose solution consumed ($F_s<0.272$, $p_s>0.605$; Figure 4D). There was a significant interaction of Sex and USV on proportion of sucrose solution consumed over 24 hours; high-calling males consumed more sucrose compared to low-calling males, and the reverse was true for females ($F_{1,36}=4.815$, $p<0.05$; interaction not depicted in figure). There were no other main effects or interactions ($F_s<1.990$, $p_s>0.167$).

3.4 ADULT PHYSIOLOGY (P76)

3.4.1 Lung Mucus, Inflammation, and Collagen—Three weeks after HDM and MCH treatments ended, AI mice had more lung mucus and inflammation than non-AI mice (mucus: $F_{1,36}=9.692$, $p<0.01$, Figure 5A; inflammation patch size: $F_{1,36}=55.51$, $p<0.001$, Figure 5B; number of inflammation patches: $F_{1,36}=41.97$, $p<0.001$; Figure 5C). AI females had more mucus than AI males (AI x Sex interaction: $F_{1,36}=4.454$, $p<0.05$). There was a trend for greater mucus production in high-USV mice ($F_{1,36}=3.587$, $p<0.10$) and a significant interaction between AI and USV ($F_{1,36}=6.151$, $p<0.05$). Within the AI group, high-calling mice produced more mucus than low-calling mice ($F_{1,24}=7.847$, $p<0.01$). There were no group differences in amount of collagen ($F_s<3.043$, $p_s>0.090$; Figure 5D).

3.4.2 Lung Cytokine Gene Expression—Three weeks after final peri-adolescent asthma treatments, *IL-4*, *IL-5*, and *IL-13* gene expression in lungs was increased in AI

compared to non-AI mice ($F_{1,22}=4.56$, $p<0.05$, $F_{1,22}=21.51$, $p<0.001$, $F_{1,22}=38.79$, $p<0.001$; Figure 5E-G). There was a significant sex difference in *IL-5* expression, with females showing greater expression than males ($F_{1,22}=14.20$, $p<0.01$). An AI x LB interaction in *IL-5* and *IL-13* expression indicated dampened expression in AI+LB mice compared to AI only mice ($F_{1,22}=4.98$, $p<0.05$; $F_{1,22}=3.82$, $p<0.10$). A main effect of USV emerged for *IL-13*, where high-calling mice had greater expression than low-calling mice ($F_{1,22}=5.60$, $p<0.05$). No other main or interaction effects occurred ($F_s<2.49$, $p_s>0.128$).

3.4.3 Circulating Basal Glucocorticoid Levels and Peripheral Circulating Cytokine Levels

—Three weeks after final asthma treatments, there was a trend for AI mice to have decreased circulating corticosterone levels compared to non-AI mice ($F_{1,37}=2.81$, $p=0.10$; Figure 6A). These results did not change when time to collect blood was included as a covariate ($F_{1,37}=2.60$, $p=.12$). AI also increased circulating IL-4 and IL-5 concentrations relative to non-AI ($F_{1,23}=13.30$, $p<0.01$; $F_{1,22}=8.29$, $p<0.01$; Figure 6B-C), with females having higher concentrations than males ($F_{1,23}=5.58$, $p<0.05$; $F_{1,22}=9.58$, $p<0.01$). The IL-13 antibody did not perform well, so results are not reported here. As control measures, circulating IL-6 and TNF- α concentrations were quantified and indicated no effects of AI, LB, Sex, or USV ($F_s<1.84$, $p_s>0.18$).

3.4.5 Brain Serotonin and Anxiety/Stress-Related Gene Expression

—Peri-adolescent LB altered adult gene expression related to serotonergic and anxiety/stress regulation. Adult LB mice had decreased brainstem *SERT* ($F_{1,37}=4.83$, $p<0.05$, Figure 6D) and increased hippocampal *5Ht1ra* and *Ctrh1* expression ($F_{1,37}=5.28$, $p<0.05$, Figure 6E; $F_{1,37}=5.08$, $p<0.05$, Figure 6F) compared to non-LB mice. There was a significant interaction of Sex, AI, and LB on *SERT* expression ($F_{1,37}=5.67$, $p<0.05$); LB males showed a trend toward downregulated *SERT* expression ($F_{1,19}=3.54$, $p<0.10$), whereas female expression was downregulated by both AI and LB ($F_{1,17}=4.23$, $p=0.055$). There were no main effects of AI, LB, Sex or USV, nor interaction effects, on hippocampal MR or GR, nor for hippocampal *Ctrh1* levels. There was a trend for peri-adolescent LB to cause increased adult MR expression ($F_{1,37}=3.12$, $p<0.10$). In PFC, a USV trend for *Ctrh1* expression in PFC indicated high-calling mice expressed more *Ctrh1* mRNA than low-calling mice ($F_{1,37}=3.83$, $p<0.10$).

4. DISCUSSION

The current study confirmed that frequent peri-adolescent intranasal house dust mite extract (HDM) treatment produced airway inflammation in a mouse model, and that this state persisted several weeks beyond the treatment period [74]. In addition, although methacholine (MCH) is often used to verify airway hyperresponsiveness [50,74,90], here, we showed that weekly peri-adolescent MCH treatments effectively induced labored breathing bouts at multiple ages throughout mouse peri-adolescence. Previous studies have demonstrated that late adolescent/early-adult respiratory allergies and inflammation lead to increased anxiety [91]. In the current study, we found that labored breathing induced by weekly MCH exposure, and not lung inflammation induced by HDM treatments, led to the most significant increases in adult anxiety-related outcomes (specifically, behavior and gene expression associated with serotonergic and CRH function). Interestingly, peri-adolescent

HDM and MCH treatment did not have a synergistic effect on adult symptoms associated with human internalizing disorders, although this might be attributed to the half MCH dose used in the HDM+MCH treated AI+LB group. These findings suggest that asthma-like repeated acute hypoxic labored breathing experiences, independent of airway inflammation, can affect physiological processes that contribute to later anxiety-related symptoms. Prior studies have established that hypoxia contributes to severity of asthma in an adult mouse model, that chronic postnatal hypoxia (P3–11) leads to increased adolescent anxiety-like behavior, and that hypoxia predicts panic disorder in asthmatic humans [50,92,93]. The current study extends these prior findings to suggest for the first time that brief, repeated asthma-like labored breathing events during mouse peri-adolescence cause changes in adult behavior and brain gene expression that are associated with an anxiety phenotype.

The current study suggests that chronic peri-adolescent asthma symptoms, which represent a real-world, common chronic stressor for many adolescents, can lead to subtle alterations in adult glucocorticoid (GC) regulation that includes young-adult basal GC upregulation with later adult downregulation. Persistent peri-adolescent lung inflammation led to dampened adult basal serum GC levels, but it did not contribute to late adolescent GC production or adult anxiety-related symptoms. In humans, experiencing asthma serves as a chronic stressor throughout development, and chronic stress is notorious for inducing GC dysregulation [20–22,28]. The current study suggests that labored breathing aspects of asthma may cause short-term alterations in GC regulation, whereas lung inflammation may lead to longer-term alterations. Further, our results indicate that peri-adolescent labored breathing causes an acute increase in basal GC production during the circadian trough in females but not in males. This is concordant with previous research demonstrating that females have higher endocrine responses than males following stress [59,84,94,95]. In humans, females are more vulnerable to developmental perturbations, and this has been associated with increased anxiety and depression rates in females compared to males [10,60]. Based on results from the current study, further investigation is needed on differential male and female late-adolescent physiological responses to acute labored breathing attacks. Sex differences in acute responses to this adolescent clinical experience may help to identify mechanisms that underlie female susceptibility to dysregulation and/or identify additional symptoms of asthma-associated internalizing disorders. The current study did not use different doses of HDM or MCH to accommodate sex differences in body weight that emerge during adolescence. Thus, future dose-response studies are required to determine if the effects observed here persist if doses are equalized for body weight.

Asthma and internalizing disorders occur at different rates in females and males. Female adolescents exhibit higher rates of asthma than male adolescents, and female adolescents and adults are hospitalized for asthma-related symptom flares more frequently than males of the same age [61–63,96,97]. Additionally, females are more sensitive to the consequences of childhood and adolescent stressors compared to males, which may contribute to higher rates of anxiety and depression in adolescent and young adult females; these female-biased effects persist into adulthood [10,58–60]. The current study, which suggests that peri-adolescent asthma symptoms lead to adult anxiety-related symptoms, sets the stage for follow-up investigations on asthma-induced anxiety-related neural substrates and sex differences in mechanisms that underlie the co-morbidity of asthma and internalizing disorders.

Serotonin function plays an important role in internalizing disorders, with decreased amounts of serotonin and reuptake ability being characteristic of depressive and anxious phenotypes [52]. Peripheral serotonin has also been demonstrated to play an important role in Th2 immune response, and recent evidence indicates that serotonin receptor agonists prevent symptoms of airway inflammation in a mouse model [98,99]. Previous human research has indicated that sex differences exist in peripheral serotonin receptor and transporter binding at baseline and in suicide victims [100,101]. In the current study, we found that adult female *SERT* expression in the brainstem was affected by both peri-adolescent HDM and MCH treatments, whereas in males this was driven by MCH treatments only. Additionally, females had a greater HDM-induced increase in Th2 cytokines in lung (*IL-5*) and serum (*IL-5* and *IL-4*) compared to males. Considering that serotonin is involved in this response and that sex differences exist in serotonin-related substrates, it is evident that our asthma treatments drove a greater female than male Th2 response in lungs and serum, and that this sex difference was not present in untreated (CON) mice. Based on results of the current and prior studies, an accentuated Th2 response in allergic responses may be one process that modulates serotonergic function, or vice versa, and that these processes may be more pronounced in females compared to males.

As previously stated, ultrasonic vocalizations (USV) rates during early postnatal life (P3-5) predict later-life temperament [66–69]. To control for different pup USV rates, indicative of an anxiety-prone phenotype, we evenly distributed high- and low-calling pups among treatment groups, and used USV call rate in statistical analyses to determine if developmental asthma affects fear-prone individuals more than others. However, we found that USV classification had no significant main or interaction effects on results in the current study. Some results indicated that experimental asthma treatments were differentially effective in high-calling relative to low-calling pups; for example, when verifying effects of HDM in the AI treatment group, high-calling (anxiety-prone) mice produced more mucus than low-calling mice. These findings suggest that in the mouse model, as with humans, early-life temperament may influence the severity of asthma symptoms later in life [102,103]. However, we found no evidence that early-life temperament exacerbated an individual's susceptibility to asthma-induced adult anxiety symptoms.

Individual housing from P50-76 is a potential limitation to the current study. Individual housing was necessary to measure individual basal corticosteroid diurnal rhythm at the end of the asthma treatments, and this housing could have affected the study results. Individual-housing is a documented stressor for rodents and has been shown to cause anxiety-like behavior [3,40,84]. The present study implemented individual housing across all experimental conditions, so any anxiety-related symptoms that resulted from this housing affected all groups. However, susceptibility to single-housing-induced anxiety may have differed among the peri-adolescent treatment groups. For example, the labored breathing mice may have had a greater anxiety-like response to single-housing than other groups. On the other hand, if single-housing increased anxiety-like behavior across all groups, the effects of our asthma treatments on adult anxiety-like symptoms may have been muted. Thus, the results presented in the current study should be interpreted with the understanding that late adolescent individual-housing may have either accentuated, muted, or otherwise

altered influences of peri-adolescent labored breathing and airway inflammation on adult behavior and physiology.

4.1 CONCLUSIONS

A validated mouse model is an essential first step for experimental studies on the independent effects of labored breathing and chronic lung inflammation on behavioral and neurobiological development. In addition, a mouse model further allows us to study bidirectional interactions of internalizing behavior and asthma. Here we documented that repeated peri-adolescent allergen and muscarinic receptor agonist exposure caused airway inflammation and labored breathing, respectively, in a mouse model. These results indicate that this model can be used to determine the independent influences of different asthma symptoms on adolescent development. More importantly, these manipulations led to changes in adult behavioral and physiological processes that have been associated with an anxiety-like phenotype. This study is the first of its kind, and while our goal was to develop a sound model for human internalizing disorders in response to peri-adolescent asthma, further investigation and improvement of this model will allow us to more confidently associate our observed results with behavior and physiology indicative of those observed in human patients. Specifically, labored breathing alone may drive increased anxiety-like symptoms in asthma patients, and chronic peri-adolescent lung inflammation may have a long-term impact on hypothalamic-pituitary-adrenal axis regulation, a risk factor for internalizing disorders. While the present study documented significant acute behavioral and neurobiological effects of peri-adolescent lung inflammation and labored breathing, further studies are necessary to: (1) address the relative permanence of these effects, (2) determine other processes affected by peri-adolescent asthma, and (3) test the influence of environmental and pharmacological interventions on minimizing adult psychiatric symptoms. Additionally, future work will require dose-response studies to elucidate the relative influence of labored breathing and airway inflammation. Based on results from the current study, the mouse model presented here provides a good foundation for follow-up studies to inform intervention, therapy, and medication regimens in young patients with asthma, with the ultimate goal of minimizing internalizing disorders and related symptoms in this population.

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ABBREVIATIONS

Y/O Years old

CON	Control (similarly handled control group)
AI	Airway Inflammation (experimental group)
LB	Labored Breathing (experimental group)
AI+LB	Airway Inflammation + Labored Breathing (experimental group)
HDM	House dust mite
MCH	Methacholine
PBS	Phosphate-buffered saline
Penh	Enhanced pause
USV	Ultrasonic vocalization
GC	Glucocorticoids
P	Postnatal Day
Th2	T helper 2
PFC	Prefrontal cortex
CT	Cycle threshold
qRT-PCR	Quantitative real time polymerase chain reaction
CRH	Corticotropin releasing hormone
GR	Glucocorticoid receptor
MR	Mineralocorticoid receptor
SERT	Serotonin transporter
5Htr1a	Serotonin receptor 1a
Crhr1	Corticotropin releasing hormone receptor 1
EPM	Elevated plus maze
SPT	Sucrose preference test
PAS	Periodic acid-Schiff stain
H&E	Hematoxylin and eosin stain
TRI	Masson's trichrome stain
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6

IL-13	Interleukin 13
TNFα	Tumor necrosis factor alpha
SPSS	Statistical Package for the Social Sciences

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HIGHLIGHTS

- Experimental conditions induced asthma-like symptoms in adolescent mice.
- Chronic adolescent asthma symptoms caused adult anxiety-related symptoms.
- Induced labored breathing raised adult anxiety behavior and brain gene expression.
- Induced airway inflammation led to decreased adult basal corticosterone production.

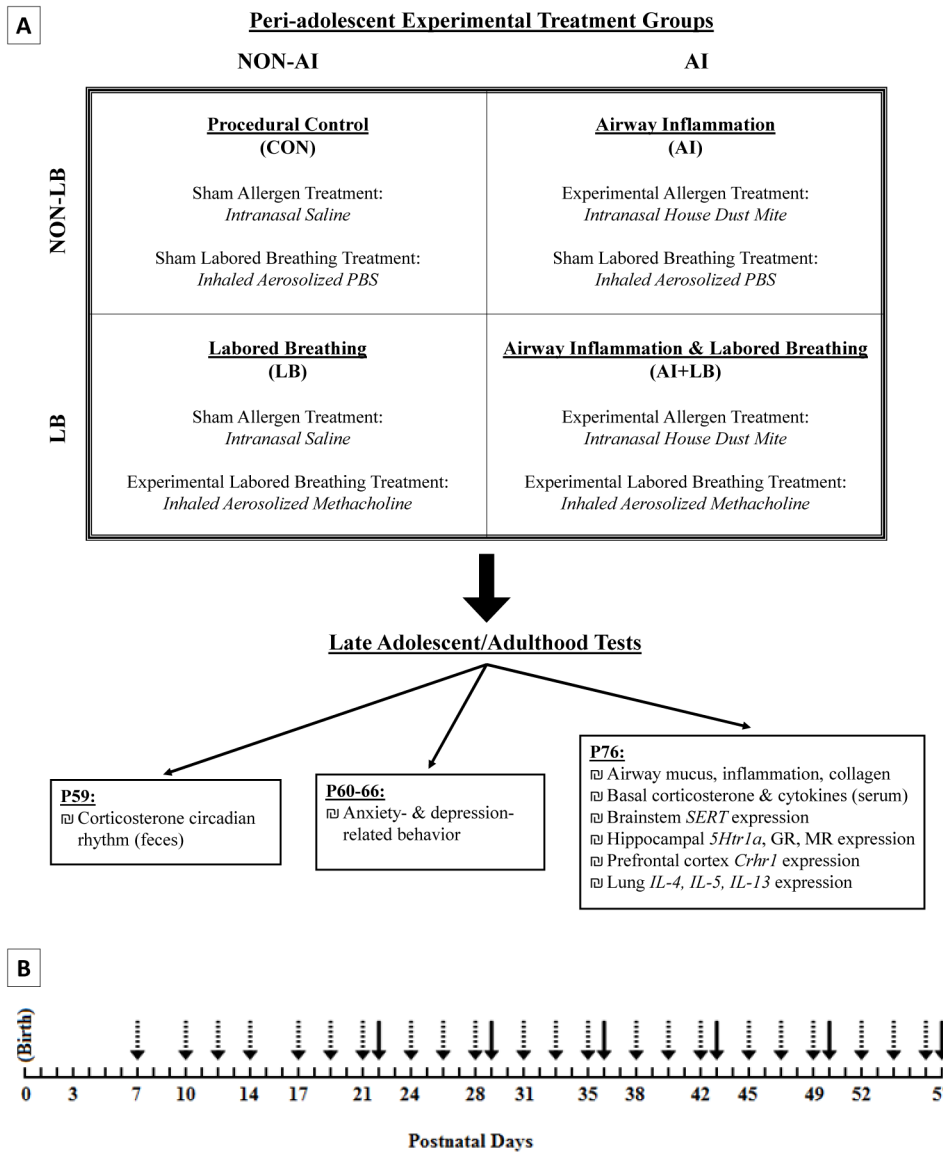


Figure 1. Study design and timelines

(A) Study design with four experimental conditions to manipulate peri-adolescent asthma symptoms to determine adult behavioral and physiological outcomes. Each experimental/control condition received a control or experimental treatment from each category (airway inflammation and labored breathing). (B) Timeline for peri-adolescent experimental manipulations. Dashed arrows indicate house dust mite (HDM) treatments to stimulate lung inflammation. Solid arrows indicate methacholine (MCH) treatments to induce labored breathing. HDM began earlier than MCH because significant inflammation requires several weeks of HDM exposure [74].

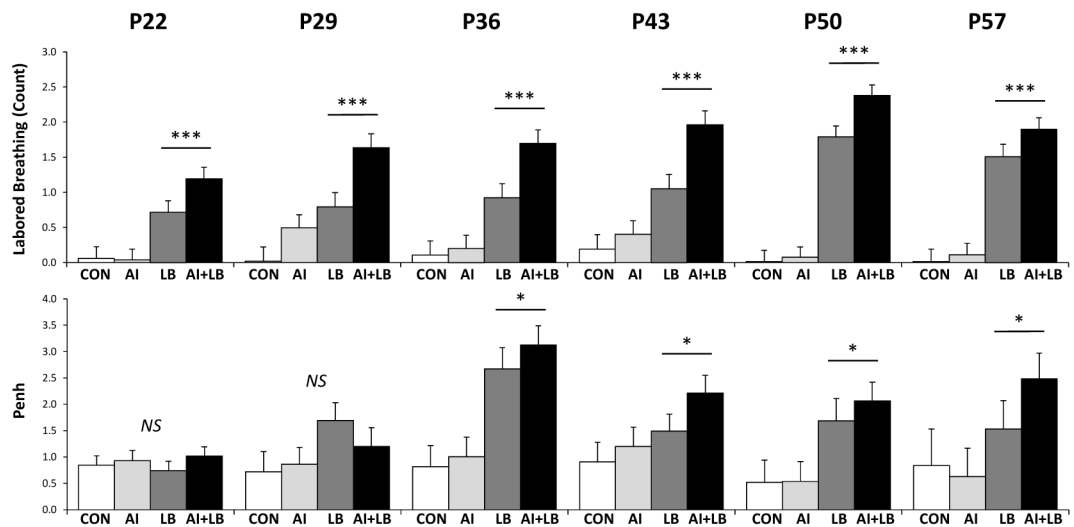


Figure 2. Peri-adolescent labored breathing and enhanced pause (Penh) values

Top: The mean number of labored breathing events during weekly methacholine (MCH) or vehicle exposure presented for the four treatment groups: Control (CON), Airway Inflammation (AI), Labored Breathing (LB), and Airway Inflammation + Labored Breathing (AI+LB). Mice that experienced MCH (LB and AI+LB) had significantly higher scores than those that did not experience MCH (CON and AI), indicated by *** ($p < 0.001$). **Bottom:** MCH treatments also caused significantly higher mean enhanced pause (Penh) values compared to saline on P36, 43, 50, and 57, indicated by * ($p < 0.05$).

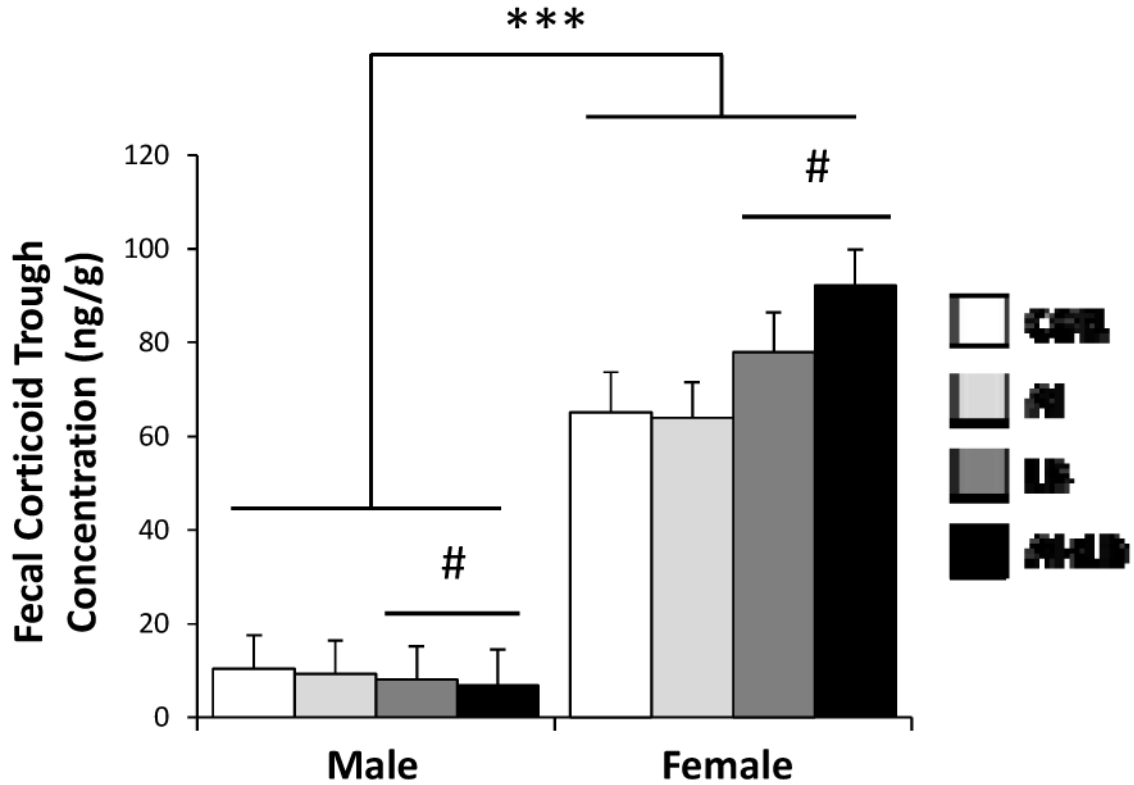


Figure 3. Late adolescent fecal corticoid concentrations

Mean fecal corticoid metabolite concentrations in late adolescent (P59) male and female mice in the four experimental groups: Control (CON), Airway Inflammation (AI), Labored Breathing (LB), and Airway Inflammation + Labored Breathing (AI+LB). Female mice had significantly greater fecal corticosteroid metabolite concentrations during the diurnal trough compared to males, indicated by *** ($p < 0.001$). Left side: Male mice that experienced MCH (LB and AI+LB groups) had a trend toward decreased fecal corticoid concentrations compared to males that did not experience MCH (CON and AI groups), indicated by # ($p < 0.10$). Right side: The reverse was true for female mice, with a trend toward increased fecal corticoid concentrations in MCH (LB and AI+LB) vs. non-MCH (CON and AI) mice, indicated by # ($p < 0.10$). Bars indicate means with standard error. [Contrasts & pairwise group comparisons: For females, there was a trend for increased fecal corticoids in AI+LB vs. CON females ($p < 0.10$) with no other group differences identified in pairwise comparisons. For males, there were no differences between individual groups.]

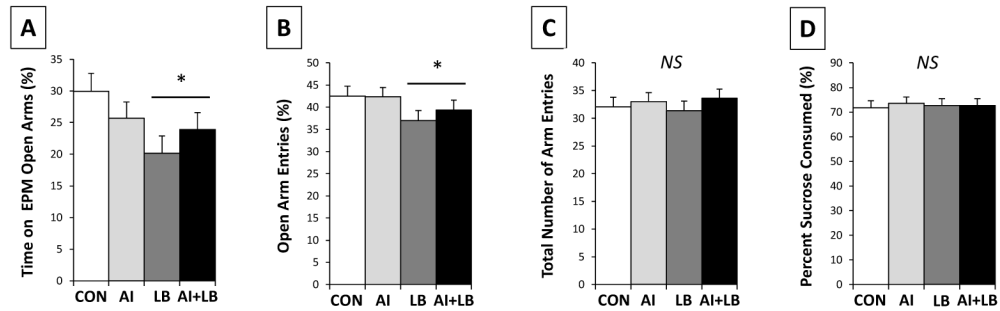


Figure 4. Adult behavior

Behavioral measures for the following four treatment groups: Control (CON), Airway Inflammation (AI), Labored Breathing (LB), and Airway Inflammation + Labored Breathing (AI+LB). Adult mouse (P60) behavior in elevated plus maze (EPM) (A–C) indicates that adolescent MCH caused increased adult anxiety-like behavior but not locomotion: there was an LB main effect on time on open arms (A) and entries into open arms (B), but not on total entries into open and closed arms (C), main effect of LB indicated by * ($p < 0.05$). No significant differences were observed in percent sucrose consumed by adult mice (P66) in sucrose preference test (SPT) (D). Bars indicate means with standard error. [*Contrasts & pairwise group comparisons*: LB and AI+LB groups spent less time on open arms of the EPM than the CON group ($p < 0.01$ and 0.05); pairwise comparisons indicated no other differences among groups.]

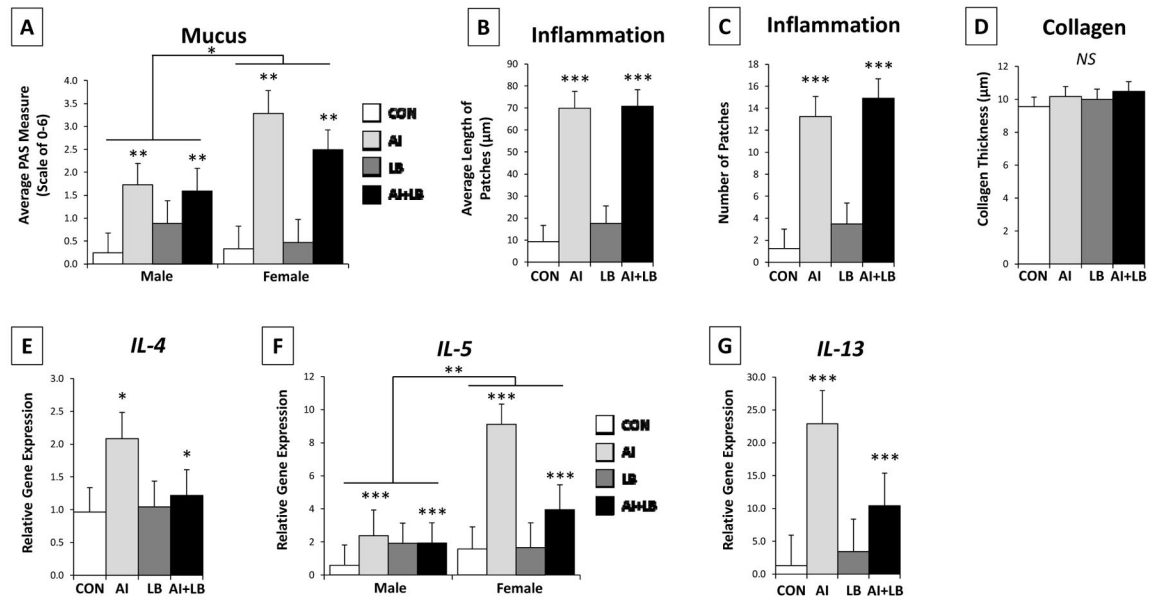


Figure 5. Adult lung state and cytokine expression

Lung function measures for the four treatment groups: Control (CON), Airway Inflammation (AI), Labored Breathing (LB), and Airway Inflammation + Labored Breathing (AI+LB). Adolescent HDM treatments (AI and AI+LB groups) led to increased mucus (A), average inflammation patch length (B), number of inflammation patches (C), and gene expression (mRNA) for *IL-4* (E), *IL-5* (F), and *IL-13* (G). Mucus and *IL-5* expression were both greater in females compared to males. Collagen measures were not significantly different across groups (D). Main effects of AI and Sex indicated by * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$). [Contrasts & pairwise group comparisons: AI and AI+LB groups had significantly more mucus ($p < 0.001$) and more inflammation (length and number of patches, $p < 0.001$) than both CON and LB groups. Compared to the CON group, AI had more gene expression of *IL-4* ($p < 0.05$), and AI and AI+LB groups had more expression of *IL-5* ($p < 0.01$ and 0.05), and *IL-13* ($p < 0.001$). Further pairwise comparisons indicated that the AI group had greater expression of *IL-5* ($p < 0.01$) and *IL-13* ($p < 0.001$) than the LB group, and the AI+LB group had greater *IL-13* expression than the LB group ($p < 0.05$.)]

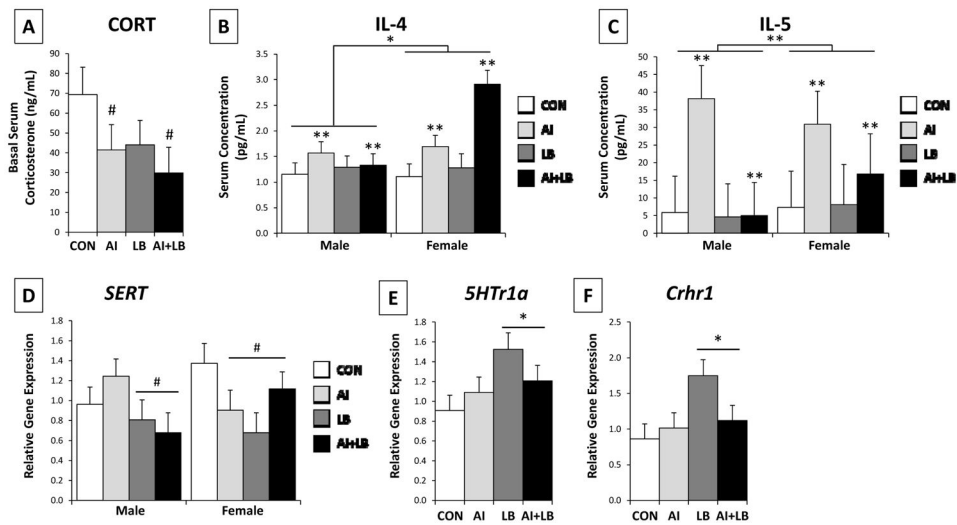


Figure 6. Adult basal corticosterone and cytokine concentrations in serum, and transporter/receptor gene expression in the brain

The four experimental groups include: Control (CON), Airway Inflammation (AI), Labored Breathing (LB), and Airway Inflammation + Labored Breathing (AI+LB). Trending effect of AI indicated by # ($p < 0.10$) for mean basal serum corticosterone concentrations (A). Main effect of AI or Sex indicated by * ($p < 0.05$), and ** ($p < 0.01$) for circulating IL-4 (B) and IL-5 levels (C). There was a main effect of LB on brainstem serotonin transporter (*SERT*) (D), hippocampal serotonin receptor 1a (*5Htr1a*) (E), and hippocampal corticotropin releasing hormone receptor 1 (*Crhr1*) (F), indicated by * ($p < 0.05$) on E and F, and excluded on D in order to show sex-specific trends of LB and AI/LB on *SERT* expression, indicated with # ($p < 0.10$). [Contrasts & pairwise group comparisons: Compared to CON mice, AI and AI+LB mice had significantly greater circulating IL-4, AI had greater IL-5 levels, and LB had significantly greater *5Htr1a* and *Crhr1* expression ($p < 0.05$ and 0.01). Pairwise comparisons revealed no other group differences.]

Table 1
Coding scheme for lung mucus

A scale from 0-6 was used to code lung mucus on PAS-stained lung slides.

Mucus Score	Description
0	No mucus or very small amounts of mucus
1	Partial thin outline of mucus on 1–2 inner airways
2	Full mucus outline around inner airway on 1–2 airway(s)
3	Full mucus outline on 3 airways
4	Mucus covering 1 entire airway OR mucus outline on 4+ airways
5	Mucus covering 1 entire airway AND mucus outline on 1+ airway(s)
6	Mucus covering 2+ airways AND mucus outline on 2+ airways

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Table 2
Statistics on the effect of peri-adolescent MCH administration on labored breathing and Penh

'Labored Breathing' column: ANOVA results for MCH main effect on labored breathing behavior at each age of administration. 'Penh' column: repeated measures ANOVA result for the effect of increasing MCH dose (time x LB interaction effect) on Penh values at each administration age. Significant results indicate a difference between LB vs. non-LB groups.

Age (Postnatal Days)	Labored Breathing	Penh
P22	$F_{1,81} = 31.03, p < 0.001$	$F_{15,86} = 0.80, p = 0.67$
P29	$F_{1,81} = 23.50, p < 0.001$	$F_{15,131} = 1.22, p = 0.26$
P36	$F_{1,81} = 32.01, p < 0.001$	$F_{15,155} = 2.74, p < 0.05$
P43	$F_{1,81} = 36.70, p < 0.001$	$F_{15,212} = 2.08, p < 0.05$
P50	$F_{1,81} = 175.87, p < 0.001$	$F_{15,137} = 1.79, p < 0.05$
P57	$F_{1,81} = 94.54, p < 0.001$	$F_{15,32} = 1.97, p = 0.05$