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## Inhaled corticosteroids as treatment for adolescent asthma: effects on adult anxiety-related outcomes in a murine model

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### Abstract

**Rationale:** Allergic asthma, typically controlled with inhaled corticosteroids (ICS), is the leading chronic health condition for youth under 18 years of age. During this peri-adolescent period, significant brain maturation occurs. Prior studies indicate that both chronic inflammation and corticosteroid medications increase risk for developing an internalizing disorder like anxiety.

**Objectives:** To determine if chronic ICS treatments exacerbate or alleviate anxiety symptoms associated with developmental allergic asthma, we used a mouse model to isolate the influence of ICS (fluticasone propionate, FLU) vs. airway inflammation (induced with house dust mite extract, HDM).

**Methods:** During development, male and female BALB/cJ mice were repeatedly exposed to HDM or saline plus one of four FLU doses (none/vehicle, low, moderate, or high). In adulthood, we assessed lung inflammation, circulating and excreted corticosteroids, anxiety-like behavior, and gene expression in stress and emotion regulation brain regions.

**Results:** FLU treatment decreased body weight and anxiety-like behavior and increased fecal corticosterone metabolite concentrations and *Crhr2* gene expression in ventral hippocampus. FLU effects were only observed in saline/non-HDM-exposed mice, and the FLU doses used did not significantly decrease HDM-induced airway inflammation. Females had greater serum and fecal corticosterone concentrations, less anxiety-like behavior, and lower *Crhr1* gene expression in ventral hippocampus and prefrontal cortex than males.

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**Conclusions:** These findings suggest that steroid medications for youth with allergic asthma may not exacerbate anxiety-related symptoms and should be avoided in children/adolescents without a health condition. The results are informative to future work on the use of corticosteroid medications during childhood or adolescent development.

### Keywords

Asthma; Inhaled corticosteroids; Adolescence; Development; HPA axis; Anxiety; Comorbidity

## 1. Introduction

One of the most common chronic health conditions that affects today's youth is asthma (8.3% of individuals under age 18), with the majority of cases characterized as allergic asthma (Li et al. 1998; Maslan and Mims 2014; Patadia et al. 2014; Akinbami et al. 2016). Early-onset asthma is typically first diagnosed in preschool-aged children, thus, chronic airway inflammation, bronchoconstriction, and steroid medication use occur during a critical period of early neurobiological development (Holder and Blaustein 2014; Patadia et al. 2014). Child and adolescent development is characterized by maturation of brain areas important for stress and emotion regulation (Spear 2000). Importantly, chronic allergic asthma is also associated with certain mental health comorbidities, including internalizing disorders like anxiety (Blackman and Gurka 2007; Katon et al. 2007; Peters and Fritz 2011; Dudeney et al. 2017). Given the significant neurobiological development that coincides with childhood asthma and associated medication use, and the significant increase in anxiety disorders in youth with asthma, it is important to determine the relative influence of asthma symptoms vs. asthma medications on anxiety-related behavioral and neurobiological processes.

Inhaled corticosteroids (ICS) are the most common maintenance drug used in patients with asthma. ICS's anti-inflammatory properties decrease lung inflammation and improve overall allergic asthma lung symptoms (De Graaf-In't Veld et al. 1995; Boulet et al. 2000; Gershman et al. 2000; Teper et al. 2004; Lee et al. 2008; Ulrich et al. 2008; Wakahara et al. 2008; Riesenfeld et al. 2010; Stempel et al. 2016). Given their effectiveness, they are prescribed at higher doses with increasing asthma severity and when lower doses do not control symptoms (Lemanske et al. 2010; Wechsler et al. 2019). ICS are deemed safe, but there are some side effects. For example, children who are administered ICS (e.g. fluticasone, budesonide) have suppressed adrenal glands and stunted growth (Ninan et al. 1993; Sim et al. 2003; Priftis et al. 2006; Guilbert et al. 2011; Kelly et al. 2012; De Leonibus et al. 2016). These side effects do not deter people from using ICS, but they can cause lasting, notable changes in developmental trajectories. The impact of ICS on asthma-related anxiety comorbidity is unclear, especially in youth. Studies focused on anxiety in youth with asthma do not always indicate patients' level of history with ICS use (Vila et al. 2000; Ortega et al. 2002; Goodwin et al. 2003; Richardson et al. 2006; Bush et al. 2007; Katon et al. 2007; Dudeney et al. 2017). Thus, it remains uncertain how ICS administration impacts anxiety symptoms in asthma patients.

Both asthma and ICS therapy are associated with altered hypothalamic-pituitary-adrenal (HPA) axis regulation, with primary adrenal insufficiency (and associated decrease in circulating glucocorticoids) as the most frequent dysregulation (Patel et al. 2001; Zaloga and Marik 2001; Zöllner 2007). Patients with asthma (with and without ICS therapy) have decreased circulating cortisol concentrations at baseline and in response to stress compared to healthy individuals (Landstra et al. 2002; Buske-Kirschbaum et al. 2003; Bakkeheim et al. 2010; Dreger et al. 2010). This low glucocorticoid secretion was also evident in mice that were developmentally exposed to house dust mite (HDM) extract to induce allergic asthma-like airway inflammation (Caulfield et al. 2017). Low circulating cortisol levels are not only a trait associated with asthma and ICS use, but also with anxiety disorders, even in children (Feder et al. 2004; Yehuda and Seckl 2011; Petrowski et al. 2013; Vreeburg et al. 2013). Additionally, corticosteroid medications have been associated with increased risk and onset of depression and anxiety in both youth and adults (Warrington and Bostwick 2006; Brown 2009). Glucocorticoids are not the only aspect of the HPA axis that is dysregulated in anxiety. Increases in corticotropin releasing hormone (CRH) and CRH receptor 1 in stress- and emotion-related brain areas have been linked to heightened anxiety-like behavior (Shepard et al. 2000; Kalin et al. 2016; Caulfield et al. 2017). Thus, HPA axis function can be altered at many levels with ICS use and in anxiety and asthma.

To determine if ICS therapy exacerbates or alleviates asthma-associated anxiety symptoms, we investigated the impact of ICS administration in a developmental chronic allergic asthma mouse model. We exposed mice to HDM (or saline control) and to one of four ICS doses (including a vehicle control dose) throughout the equivalent of child and adolescent development (Caulfield et al. 2017, 2018). In the current study, we administered the ICS medication fluticasone propionate (FLU), which is a lipid-soluble corticosteroid often used for long-term asthma management, and it has been shown to decrease eosinophilic lung inflammation in airways and bronchoalveolar lavage fluid in both humans and animal models (Johnson 1995; Lee et al. 2008; Ulrich et al. 2008; Wakahara et al. 2008; Riesenfeld et al. 2010). The study design allowed us to evaluate potential independent and synergistic effects of ICS and allergic asthma. We measured inflammation and anxiety-related outcomes in young adulthood after mice were no longer exposed to HDM and/or FLU. *A priori*, we hypothesized that FLU would lead to reduced airway inflammation and body growth, as well as greater adrenal suppression, anxiety-like behavior, and brain anxiety-related gene expression in a dose-dependent manner in HDM-exposed mice.

## 2. Materials and methods

### 2.1 Study design

To elicit airway inflammation, half of the experimental mice were exposed to chronic intranasal house dust mite extract (HDM, n=59), and the other half received saline control (n=57). Within the HDM and saline groups, mice were further distributed into one of four chronic intranasal fluticasone propionate (FLU) dose groups (vehicle, low, moderate, high dose; n=28–30 per group). Male and female mice were evenly distributed across all treatment groups. To determine the effects of FLU alone and the effects of FLU in combination with allergic asthma, we analyzed saline control and HDM-exposed mice

separately (6–8 males and females in each FLU dose group within saline and within HDM groups).

## 2.2 Mouse breeding and housing

A timeline of procedures is outlined in Figure 1. Male and female BALB/cJ breeders were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in a Pennsylvania State University vivarium in three cohorts (19 litters, 24–57 pups per cohort, 58 male and 58 female offspring). Lights were maintained on a 12:12 light:dark cycle with lights OFF at 10:00 hr. The vivarium was maintained at 72±2°F, and all mice had *ad libitum* access to food and water throughout the study. Pups were identified with non-toxic Sharpie® markings until they received unique ear punches on postnatal day (P) 9. Ultrasonic vocalizations (USV) were recorded at 65 Hz using the “Isolation” method: pups were individually put into an empty cage for two minutes per day from P3–5 to measure USVs, and then they were returned to the home cage. Calls were counted using Avisoft-SASLab Lite (Avisoft Bioacoustics). Pups were considered “high-calling” or “low-calling” based on the average number of calls relative to the litter median, and equal numbers of high- and low-calling pups were distributed across all experimental groups to control for pre-manipulation anxiety-prone phenotypes (Dichter et al. 1996; Brunelli et al. 1997; Branchi et al. 2001; Hahn and Lavooy 2005; Caulfield et al. 2017, 2018). Mice were weaned from the mother at P21 and placed into groups of 2–3 same-sex mice, keeping littermates together as best as possible. Each cage contained at least one HDM-treated and one saline control mouse, and mice within a single cage were exposed to the same dose of FLU. Mice remained in these housing conditions/groups until sacrificed at P69, two weeks after chronic developmental allergen and inhaled corticosteroid exposure was complete (described below). All work was approved by the Pennsylvania State University Institutional Animal Care and Use Committee and abides by the National Institutes of Health guide for the care and use of laboratory animals.

## 2.3 Allergen administration

Allergen administration was performed as previously described (Caulfield et al. 2017, 2018). Briefly, mice were lightly anesthetized with isoflurane. Mice were exposed to intranasal house dust mite extract (*Dermatophagoides pteronyssinus*, HDM; Greer Labs, NC, USA) three times per week (P7–16: 10µg protein in 10µL saline, P17–56: 15µg protein in 15µL saline) or an equivalent volume of saline (Sagiani et al. 2009; Caulfield et al. 2017, 2018). Body weights were collected at each treatment.

## 2.4 Inhaled corticosteroid administration

Five milligrams of fluticasone propionate powder (FLU; F9428–5MG, Sigma-Aldrich) was obtained and dissolved in 70% ethanol. To create the desired doses, the stock FLU was combined with 70% ethanol and saline such that all doses had 3.5% ethanol (Lee et al. 2008). Mice were treated with 20µL of one of four doses, and the doses are referred to throughout the rest of the manuscript in the following manner: 0µg/µL (vehicle), 0.25µg/µL (low dose), 0.5µg/µL (moderate dose), or 1.0µg/µL (high dose). These doses were identified based on prior studies that used FLU in mice and were adjusted to take into consideration total weekly FLU exposure and average BALB/cJ mouse weight during development (Lee

et al. 2008; Ulrich et al. 2008; Wakahara et al. 2008; Riesenfeld et al. 2010). From P24–56, mice were treated with FLU immediately after HDM/saline. FLU administration began on P24, which was two weeks after HDM administered began, to allow for sufficient time to promote detectable HDM-induced inflammation (Saglani et al. 2009). We used multiple doses to identify the most effective dose to minimize inflammation in adolescent mice and to test potential dose-dependent effects on adult anxiety-related outcomes.

## 2.5 Corticosterone analyses

To measure basal corticosterone secretion, we analyzed both fecal and serum samples. On P59, 3 days after completion of HDM/FLU/saline exposure, fecal samples were collected between 14:00–15:00 hr. This sample collection time was used to estimate basal circulating corticosterone concentration during the morning circadian peak, given the ~4-hr time lag for circulating steroids to be metabolized and excreted in feces (Touma et al. 2004). Samples were stored at –80°C until corticosterone (CORT) metabolites were extracted and measured. Some samples had to be combined based on sex, HDM, and FLU treatment to achieve a minimum dry fecal weight for analysis (final n at analysis=79). Samples were extracted using previously described methods (Cavigelli et al. 2005, 2006; Thanos et al. 2009). Blood samples were collected at sacrifice (P69) via cardiac puncture between 14:00 – 17:00 hr. This collection time was used to estimate afternoon basal corticosterone concentration (levels between the daily peak and trough). Samples were centrifuged for 15 minutes at 12,000g at 4°C, and serum was collected and stored at –80°C. CORT and metabolites were measured from fecal extracts and serum using a commercial [125I] radioimmunoassay kit (MP Biomedicals, Solon, OH, USA) following the manufacturer guidelines. Intra- and inter-assay coefficients of variance were 6.24 and 7.73 (for low control) and 6.09 and 4.70 (for high control).

## 2.6 Lung inflammation histology

At sacrifice on P69, two weeks after HDM and FLU exposures were completed, a sample of lung tissue from the left and right lobes was collected immediately into formalin. Each sample was embedded in paraffin, sliced, and stained with hematoxylin and eosin (H&E). The number of inflammation patches (clusters of inflammatory cells) and the size of each inflammation area perpendicular to an airway/vessel membrane (length of 20µm diameter or larger) were measured as previously described (Caulfield et al. 2017, 2018). The total number of inflammation patches and the mean lengths of these areas were calculated from the six largest areas (three patches per lung sample per mouse, or as many as possible).

## 2.7 Anxiety-like behavior

The elevated plus maze (EPM) is a classic test to assess rodent anxiety-like behavior (Pellow et al. 1985; Lister 1987; Hogg 1996; File 2001; Carobrez and Bertoglio 2005). Adult mice were tested on the EPM on P62 for 5 minutes. The maze consisted of two open arms (30 × 5 cm) and two closed arms (30 × 14.5 × 5 cm) that were elevated off the ground by 42 cm. The EPM tests were conducted between 14:00 – 17:00 hr in infrared light (~ 2 lux). Percent of time spent on and percent of entries into open arms of the EPM were considered as measures of unconditioned anxiety (Rodgers and Dalvi 1997; File 2001). Closed arm entries were recorded as a measure of locomotion (Rodgers and Dalvi 1997; File 2001).

Mice were recorded with an overhead video camera and tracked with an automated tracking system (Any-maze v.4.60, Stoelting, Wood Dale, IL, USA). Entry into an arm was defined as 85% of the mouse body passing over an arm threshold.

## 2.8 Gene expression

Tissues collected by dissection at sacrifice (lungs, hippocampus, prefrontal cortex [PFC]) were stored at  $-80^{\circ}\text{C}$ . RNA was isolated from tissue using TRIzol<sup>TM</sup> (Invitrogen, USA) following manufacturer guidelines, and concentrations were determined with a NanoDrop<sup>TM</sup> spectrophotometer (Thermo Fisher Scientific, USA). High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, USA) were used to reverse transcribe cDNA from RNA. Quantitative real time PCR was used to measure the relative abundance of genes using the standard  $2^{-\Delta\Delta\text{CT}}$  method. These procedures have been used previously (Caulfield et al. 2017, 2018). Gene sequences used for PCR were obtained from NCBI GenBank and included: beta-actin (forward: 5'-GCCCTGAGGCTCTTTTCC-3', reverse: 5'-TGCCACAGGATTCCATACCC-3'), interleukin 5 (IL-5; forward: 5'-ACAGACATGCACCATTGCCA-3', reverse: 5'-TGGGTTCCATCTCCAGCACT-3'), corticotropin releasing hormone (CRH; forward: TACCAAGGGAGGAGAAGAGAGCG, reverse: CAAGGCAGGCAGGACGACAG), CRH receptor 1 (Crhr1; forward: 5'-GTGCTTCCCATGTTTTGGAT-3', reverse: 5'-TCGTGTGAAGCCTTGGGTTT-3'), CRH receptor 2 (Crhr2; forward: CCGTGCCCCGAGTACTTCAA, reverse: CCACGGAAACACAGTGACCCA), and glucocorticoid receptor (GR; forward: GCTTGTGGGGTGGATTCCGG, reverse: AGTCCATCACGCTTCCCCTC).

## 2.9 Statistical analyses

To verify that chronic developmental HDM exposure caused lasting lung inflammation in adulthood, lung inflammation patches (count and length) and *IL-5* gene expression were analyzed with an analysis of variance (ANOVA) with asthma group (HDM, saline control) as a factor. As previously reported, in the current study we found that airway inflammation was induced, and lung inflammation patch count, patch length, and *IL-5* gene expression were correlated with one another (Table 1) (Caulfield et al. 2017, 2018).

To focus on FLU effects in mice with significant airway inflammation vs. FLU effects alone, all remaining analyses were conducted for HDM-exposed and saline control mice separately. Factors in the separate ANOVAs were sex (male, female) and FLU group (vehicle, low, moderate, high dose). If main effects or interactions with sex were observed  $p < 0.10$ , further analyses were conducted separately for males and females. We report  $F$  values with degrees of freedom. Significant results were  $p < 0.05$  and marginal effects were  $p < 0.10$ . Results from these separate ANOVAs with HDM- vs. saline-exposed mice did not differ from omnibus ANOVAs with all animals and three factors (sex, FLU, HDM). For clarity we report results of the separate ANOVAs for HDM- vs. saline-exposed mice.

To determine if cohort contributed significant variance to the results, we included cohort as a factor in all ANOVAs, and if the cohort factor had an effect of  $p < 0.10$ , then a cohort mean was calculated for the outcome variable and included as a covariate in the analysis. In figures, estimated marginal means are presented if analyses required a cohort mean covariate

– control: inflammation patch length, inflammation patch count, % open arm time, % open arm entries, closed arm entries, ventral hippocampus gene expression (*CRH*, *Crhr1*, *Crhr2*, *GR*), and prefrontal cortex gene expression (*Crhr2*); HDM: lung gene expression (*IL-5*), % open arm time, % open arm entries, ventral hippocampus gene expression (*CRH*, *Crhr1*, *Crhr2*, *GR*), and prefrontal cortex gene expression (*Crhr2*).

To identify possible pathways by which FLU may affect anxiety-like behavior we conducted correlational analyses to identify linear relationships between anxiety-related outcomes. We compared anxiety-related behaviors (percent open arm entries, percent open arm time on EPM) to serum CORT, and ventral hippocampus gene expression (*CRH*, *Crhr1*, *Crhr2*, *GR*) to serum CORT and anxiety-related behavior. Because there were significant sex differences in many of these variables, we conducted these analyses separately for males and females. We reported Pearson's correlation coefficient (*r*), degrees of freedom, and *p* value.

To measure effects of FLU on body weight gain during development, we analyzed overall change in weight from P24 to P56 with an ANOVA (sex and FLU as factors), and conducted a repeated measures ANOVA to analyze weekly change in body weight during this time. To determine if body weight recovered after the FLU treatment period, we analyzed change in weight from P56 to P69 with an ANOVA (sex and FLU as factors).

To obtain a normal distribution, some variables were transformed (natural log transformation): inflammation patch length, inflammation patch count, *IL-5* lung gene expression, serum corticosterone, fecal corticosterone, % open arm time, % open arm entries, ventral hippocampus and prefrontal cortex gene expression (*CRH*, *Crhr1*, *Crhr2*, *GR*). In all figures, untransformed means are displayed.

### 3. Results

#### 3.1 Lung analyses

**3.1.1 Lung histology**—Lung inflammation was greater in females compared to males, but it was not affected by FLU treatments. In HDM-exposed mice, females had more and larger inflammation patches than males (main effect of sex for patch count:  $F_{1,51}=15.0$ ,  $p<0.001$ ; Figure 2a; patch length:  $F_{1,51}=10.4$ ,  $p<0.01$ ; Figure 2b). No other significant effects were observed on inflammation patch count or length, even when males and females were analyzed independently ( $F_s<2.0$ ,  $p>0.141$ ).

**3.1.2 Lung gene expression**—Gene expression of *IL-5* in the lungs was elevated in females compared to males, and it was unaffected by FLU treatments. In both HDM-exposed and saline control mice, females had greater gene expression of *IL-5* in lungs than males (main effect of sex, HDM:  $F_{1,50}=7.4$ ,  $p<0.01$ , control:  $F_{1,48}=7.5$ ,  $p<0.01$ ; Figure 2c). No other significant effects were observed for *IL-5* gene expression, even when males and females were analyzed independently ( $F_s<1.3$ ,  $p>0.278$ ).

#### 3.2 Body weight change

Change in body weight from P24 (FLU initiation day) are shown for P31, P38, P45, P52, and P56 during the treatment period (Figure 3a). Repeated measures ANOVAs indicated that

males gained more weight than females (main effect of sex, HDM:  $F_{1,50}=243.9$ ,  $p<0.001$ , control:  $F_{1,49}=174.5$ ,  $p<0.001$ ). In saline controls, there was a main effect of FLU, where mice exposed to the moderate or high FLU doses gained less weight during the treatment period than vehicle-treated mice ( $F_{1,49}=3.4$ ,  $p<0.05$ ; paired comparisons: 0 vs. 0.5µg/µL,  $p<0.05$ ; 0 vs. 1.0µg/µL,  $p<0.01$ ; 0.25 vs. 1.0µg/µL,  $p<0.10$ ). These results were primarily driven by females; in saline control females, there was a main effect of FLU, where females exposed to the moderate or high FLU dose gained less weight during treatment than vehicle and low-dose FLU females ( $F_{1,26}=4.6$ ,  $p<0.05$ ; paired comparisons: 0 vs. 0.5µg/µL,  $p<0.01$ ; 0 vs. 1.0µg/µL,  $p<0.05$ ; 0.25 vs. 0.5µg/µL,  $p<0.01$ ; 0.25 vs. 1.0µg/µL,  $p<0.05$ ). No other significant effects were observed with the repeated measures ANOVA overall or separately for males and females ( $F_s<1.8$ ,  $p>0.163$ ).

For mean body weight change from first to last day of treatment, males gained more weight than females, and mice treated with FLU gained less weight than vehicle-treated mice. In both HDM-exposed and saline control mice, males gained more weight than females (main effect of sex, HDM:  $F_{1,51}=268.0$ ,  $p<0.001$ , control:  $F_{1,48}=228.8$ ,  $p<0.001$ ; Figure 3b). In saline control mice, there was a marginal FLU effect, where those exposed to the moderate and high FLU dose gained less weight than those exposed to vehicle ( $F_{3,48}=2.7$ ,  $p=0.059$ ; paired comparison:  $p<0.05$ ). Much of this effect was driven by females. In saline control females, there was a main effect of FLU dose, where the moderate FLU group gained less weight than the vehicle and low-dose groups ( $F_{3,25}=3.2$ ,  $p<0.05$ ; paired comparison:  $p<0.05$ ; Figure 3b). No other significant effects were observed for weight gain during the FLU treatment period ( $F_s<1.8$ ,  $p>0.170$ ). After the treatment period, there were no significant differences in weight gain in either HDM-exposed or saline control mice ( $F_s<0.9$ ,  $p>0.433$ ; Figure 3c).

### 3.3 Corticosterone levels

**3.3.1 Fecal corticosterone metabolites**—In feces, females had higher concentrations of CORT metabolites than males, and FLU administration increased CORT metabolite concentrations. In both HDM-exposed and saline control mice, females produced more CORT compared to males (main effect of sex, HDM:  $F_{1,32}=425.5$ ,  $p<0.001$ , control:  $F_{1,31}=686.3$ ,  $p<0.001$ ; Figure 4a). HDM-exposed mice also demonstrated a sex by FLU dose interaction, where those exposed to the moderate dose of FLU had the highest CORT of all female groups and the lowest CORT of all male groups ( $F_{1,32}=6.0$ ,  $p<0.01$ ). In saline control mice, there was a marginal effect of FLU dose where those exposed to the moderate dose had greater CORT production than vehicle-exposed mice ( $F_{1,31}=2.3$ ,  $p<0.10$ ; paired comparison:  $p<0.05$ ). In females, there was a main effect of FLU dose in both HDM-exposed and saline control groups (HDM:  $F_{3,18}=5.5$ ,  $p<0.01$ , control:  $F_{3,18}=4.8$ ,  $p<0.05$ ). In HDM-exposed females, those administered the moderate FLU dose produced more CORT than vehicle and low-dose females (paired comparisons: 0 vs. 0.5µg/µL,  $p<0.05$ ; 0.25 vs. 0.5µg/µL,  $p<0.001$ ; Figure 4a). In saline control females, all FLU-dose groups produced more CORT than the FLU vehicle group (paired comparisons: 0 vs. 0.25µg/µL,  $p<0.05$ ; 0 vs. 0.5µg/µL,  $p<0.01$ ; 0 vs. 1.0µg/µL,  $p<0.01$ ; Figure 4a). No other significant effects were observed ( $F_s<1.694$ ,  $p>0.214$ ).

**3.3.2 Serum corticosterone**—In both HDM-exposed and saline control mice, females had higher serum CORT compared to males (main effect of sex, HDM:  $F_{1,51}=17.3$ ,  $p<0.001$ , control:  $F_{1,49}=16.1$ ,  $p<0.001$ ; Figure 4b). No other significant effects were observed in HDM-exposed or saline control mice ( $F_s<2.0$ ,  $p>0.136$ ).

### 3.4 Anxiety-like behavior

FLU administration resulted in decreased anxiety-like behavior on the EPM in both male and female saline-treated mice. In saline-treated mice, there was a main effect of FLU dose, where mice given the highest dose made more entries on the open arms than all other doses ( $F_{3,48}=4.2$ ,  $p<0.05$ ; paired comparisons: 1.0 vs. 0 $\mu$ g/ $\mu$ L,  $p<0.10$ ; 1.0 vs. 0.25 $\mu$ g/ $\mu$ L,  $p<0.01$ ; 1.0 vs. 0.5 $\mu$ g/ $\mu$ L,  $p<0.05$ ; Figure 5a). HDM-exposed females made marginally more entries onto the open arms compared to HDM-exposed males (main effect of sex,  $F_{1,50}=3.9$ ,  $p=0.053$ ; Figure 5a). No other significant effects were observed for percent open arm entries ( $F_s<0.8$ ,  $p>0.370$ ). No significant effects were observed for percent open arm time ( $F_s<2.0$ ,  $p>0.162$ ; Figure 5b). Last, HDM-exposed males made more entries onto closed arms compared to HDM-exposed females ( $F_{1,50}=4.6$ ,  $p<0.05$ ). No other significant effects were observed in closed arm entries ( $F_s<1.7$ ,  $p>0.182$ ; Figure 5c).

In both males and females, percent open arm entries were positively correlated with percent open arm time (male:  $r=0.894$ ,  $df=56$ ,  $p<0.001$ , female:  $r=0.898$ ,  $df=56$ ,  $p<0.001$ ). In females, basal serum CORT levels were positively correlated with percent open arm entries and time (Table 2).

### 3.5 Brain gene expression

**3.5.1 Ventral hippocampus**—In the ventral hippocampus, there were no significant differences in *CRH* gene expression ( $F_s<0.7$ ,  $p>0.400$ ; Figure 6a). In saline-treated mice, females expressed less *Crhr1* (main effect of sex:  $F_{1,48}=7.2$ ,  $p<0.01$ ; Figure 6b), less *Crhr2* (main effect of sex:  $F_{1,48}=8.1$ ,  $p<0.01$ , Figure 6c), and marginally less *GR* (main effect of sex:  $F_{1,48}=3.8$ ,  $p<0.10$ ; Figure 6d) than males. In saline-treated males, there was a main effect of FLU dose on *Crhr2* gene expression, where mice exposed to the moderate FLU dose had higher gene expression than vehicle and low dose males ( $F_{3,22}=3.7$ ,  $p<0.05$ ; paired comparisons: 0 vs. 0.5 $\mu$ g/ $\mu$ L,  $p<0.01$ ; 0.25 vs. 0.5 $\mu$ g/ $\mu$ L,  $p<0.01$ ; Figure 6c). No other significant differences were observed for *Crhr1* or *Crhr2* or *GR* gene expression ( $F_s<1.5$ ,  $p>0.223$ ).

In both males and females, serum CORT levels were negatively correlated with ventral hippocampus *CRH* and marginally negatively correlated with *Crhr1* expression. In addition, for females, serum CORT was also negatively correlated with ventral hippocampus *GR* expression (Table 3, upper half titled ‘All Groups’). In males, ventral hippocampus *Crhr1* and *Crhr2* expression were positively correlated with percent open arm entries, and in females, *Crhr2* expression was marginally positively correlated with percent open arm entries (Table 3, upper half titled ‘All Groups’).

Because FLU treatments affected anxiety-related behavior only in saline-treated mice, we conducted similar correlations for saline-treated mice only. In these males, but not

females, serum CORT was still negatively correlated with *CRH* and *Crhr1* expression; in females, serum CORT was marginally positively correlated with ventral hippocampal *Crhr2* expression (Table 3, lower half titled ‘Saline-treated control mice’). (For females, but not males, serum CORT was still positively correlated with open arm entries,  $r=0.364$ ,  $p=.048$ ,  $df=28$ .) In both males and females, the only ventral hippocampus expression still associated with anxiety-related behavior was *Crhr2*, which was positively correlated with percent open arm entries (Table 3, lower half titled ‘Saline-treated control mice’).

**3.5.2 Prefrontal cortex**—In HDM-exposed mice, there was a marginal interaction of sex and ICS dose for prefrontal cortex *CRH* gene expression, where the low FLU dose had opposite effects on male vs. female *CRH* expression ( $F_{3,51}=2.5$ ,  $p<0.10$ , not shown in figure). No other significant effects were observed for *CRH* gene expression ( $F_s<2.0$ ,  $p>0.133$ ). Females expressed less *Crhr1* than males (main effect of sex, HDM:  $F_{1,51}=3.3$ ,  $p<0.10$ , saline:  $F_{1,49}=5.7$ ,  $p<0.05$ ). No other significant effects were observed for *Crhr1* gene expression ( $F_s<2.1$ ,  $p>0.107$ ). There were no significant effects observed for *Crhr2* ( $F_s<2.0$ ,  $p>0.133$ ) or *GR* gene expression ( $F_s<1.4$ ,  $p>0.265$ ). PFC gene expression was not correlated to any anxiety-related measures.

## 4. Discussion

In the present study, we tested whether ICS (specifically fluticasone propionate, FLU) exacerbated or diminished anxiety-related behavior and physiology in a developmental asthma mouse model. The present study is unique because we focused on allergen (HDM) and corticosteroid (FLU) administration during a period of mouse development that is equivalent to human childhood and adolescence. This is a clinically-significant developmental period because asthma is more prevalent in children than adults (Akinbami et al. 2012). Consistent with prior work, in the current study we demonstrated that chronic exposure to HDM during development caused increased inflammatory gene expression (*IL-5*) and greater inflammation in lungs 13 days after final allergen exposure (Sagiani et al. 2009; Caulfield et al. 2017, 2018). Additionally, FLU administration during this time affected body weight gain, adult anxiety-like behavior, glucocorticoid production, and *Crhr2* expression in ventral hippocampus. Most of these results were primarily in mice that did not have allergen-induced airway inflammation (i.e. saline controls).

ICS medications are typically prescribed as a therapy to help regulate inflammatory symptoms of asthma. Evidence suggests that as little as one week of ICS therapy is sufficient to reduce inflammation and improve breathing in children and adults with asthma (Teper et al. 2004; Fukakusa et al. 2005; Cowan et al. 2010; Keskin et al. 2016). These same effects have been observed in rodent models of adult asthma – exposure to an allergen plus ICS medication reduced inflammatory cells (mainly eosinophils) in lung fluid, decreased smooth muscle growth in airways, and reduced airway mucus buildup (Joad et al. 2008; Lee et al. 2008; Ulrich et al. 2008; Wakahara et al. 2008; Riesenfeld et al. 2010). In the present study, we expected that chronic exposure to ICS during development would reduce adult lung inflammation in a dose-dependent manner. We did not observe a statistically significant FLU-induced decrease in lung inflammation for mice exposed to chronic HDM. We may have underestimated the FLU dose needed to decrease inflammation

during development and that greater effects would have been observed with higher doses. Alternatively, increasing the dose at a later age may have led to larger overall effects of FLU by the end of the treatment period. Clinically, it is common for children with uncontrolled asthma to be prescribed higher ICS doses and/or additional medications (e.g. long-acting beta agonists) over time to better regulate symptoms (Lemanske et al. 2010; van Aalderen et al. 2015; Wechsler et al. 2019). In the present study, we based our doses on average adolescent mouse body weights, although clinically, children are prescribed doses of ICS medication based on symptomology and typically without consideration of body weight (National Heart Lung and Blood Institute 2012). Other studies suggest that treatment with ICS should be combined with another form of medication to help further decrease symptoms (Lemanske et al. 2010; Riesenfeld et al. 2010; O'Byrne et al. 2014). Future studies should examine the impact of combined medications, adjusting dosage by body weight, or changing ICS dosage as mice age in the developmental mouse model of asthma.

While the goal of ICS medications is to regulate inflammatory symptoms and improve lung function in asthma patients, they can have side effects that are critical to consider for youth. Importantly, chronic ICS administration affects bone growth such that children who take ICS medications grow less and grow slower than their peers, and these effects appear stronger with FLU treatment vs. other ICS types (Merkus et al. 1993; Hanania et al. 1995; Guilbert et al. 2011; Kelly et al. 2012; De Leonibus et al. 2016). In the present study, we found that body weight gain during development was affected by FLU exposure in saline control mice. For example, females given a moderate and high FLU dose gained 18 and 12% less weight than females treated with FLU vehicle. These effects on body weight suggest that the FLU doses used in the current study were sufficient to affect development, although they did not significantly decrease lung inflammation. Interestingly, for the group with allergic asthma-like lung inflammation (i.e. HDM-exposed), we did not observe a significant decrease in body weight as a result of chronic FLU treatments. Prior studies with non-human primates indicated that ICS exposure in the absence of asthma can be detrimental and may result in increased lung inflammation (Joad et al. 2008). In the current study with mice, the decrease in body weight after FLU exposure in the absence of allergen indicates that unnecessary ICS exposure during development can have negative impacts.

In the current study, body weight change after HDM/FLU exposure did not indicate compensatory increases in body weight following the treatment period. These results suggest that decreased growth due to chronic FLU exposure during development may cause lasting effects into adulthood. Prior research has demonstrated increased weight gain during the weeks immediately after a chronic stressor or manipulation during adolescence. Some of these cases were whole body weight increases driven by increased food intake, while others were organ-specific weight increases (Lee et al. 2010; Goto et al. 2014; Smith et al. 2018). The current results suggest that ICS treatments during development may have longer lasting impacts on growth and body weight compared to other challenges experienced during the developmental period.

Clinical literature indicates that another side effect of chronic ICS use in asthma patients is adrenal suppression, typically assessed with serum cortisol (Sim et al. 2003; Priftis et al. 2006, 2008). Anxiety is prevalent in patients with asthma (Vila et al. 2000; Goodwin et al.

2003; Dudeney et al. 2017), and people who exhibit anxiety and depression also demonstrate lower serum and salivary cortisol/cortisol awakening response compared to healthy controls (Jarcho et al. 2013; Vreeburg et al. 2013; Vaccarino et al. 2015). Cortisol/CORT can be measured in different media, such as blood and saliva to examine acute changes in hormone secretion, or in urine, feces, or hair to examine cumulative hormone production (Touma et al. 2003; Caulfield et al. 2017; Caulfield and Cavigelli 2020). In the present study, we examined basal CORT production from fecal and serum samples. Females had higher levels of CORT in both serum and feces compared to males, regardless of any treatments. This has been observed previously for both serum and fecal CORT measures in mice (Aoki et al. 2010; Caulfield et al. 2017). In addition, in the current study, fecal CORT metabolite levels that represented circulating CORT at the beginning of the active period were elevated three days after chronic FLU treatments in mice that received a moderate FLU dose, but within 2 weeks these differences were not present in afternoon basal serum CORT concentrations. Human adrenal insufficiency is typically assessed by measuring basal cortisol levels and cortisol in response to an adrenocorticotropin hormone (ACTH) challenge. In asthmatic children on ICS medications, adrenal insufficiency has been characterized as low basal cortisol but also suppressed cortisol response to ACTH (Patel et al. 2001; Zöllner 2007), and asthma patients that discontinue ICS therapy may return to normal cortisol responses to ACTH (Patel et al. 2001). The results of the current study suggest that the chronic steroid use may cause short-term but maybe not long-term effects on basal CORT levels. We did not measure CORT response to ACTH to know if this effect reflects true adrenal insufficiency. Interestingly in females, basal serum CORT was positively correlated with open arm time/entries on the EPM, indicating that lower circulating CORT was associated with increased anxiety-like behavior. This finding has been observed in humans where patients with depression and anxiety have lower salivary and serum cortisol concentrations (Feder et al. 2004; Yehuda and Seckl 2011; Petrowski et al. 2013; Vreeburg et al. 2013). We also found that like in females, male saline controls that were exposed to the highest FLU dose demonstrated less anxiety-like behavior than all other FLU groups. Although for males, this decrease in anxiety-like behavior with FLU treatment occurred in the absence of any measured changes in CORT. These results suggest that basal glucocorticoid levels may not mediate anxiety-related outcomes that result from ICS treatment during development, or that this mediation may be specific to females.

In the present study, we measured gene expression in brain areas associated with regulation of the HPA axis, emotions, and internalizing disorders (McEwen and Stellar 1993; Binder and Nemeroff 2010; Gray et al. 2015). We also examined relationships between gene expression in these areas and anxiety-like behavior and serum CORT. We did not observe any changes in *CRH* or *GR* gene expression in either the ventral hippocampus or PFC in any experimental groups. However, we did observe correlations in ventral hippocampus gene expression and both serum CORT and anxiety-like behavior. In both males and females, those with lower CORT had higher *CRH* expression, and in females, higher *GR* gene expression, suggesting compensatory expression of HPA-axis regulatory genes when CORT production is low. Also, decreased anxiety-like behavior was associated with higher ventral hippocampus (but not PFC) *Crhr1* and *Crhr2* expression. This appears to be in contrast to prior studies that have shown decreased anxiety-like behavior in limbic/forebrain *Crhr1*

knockout mice (Müller et al. 2003), however *Crhr1* can be involved in both anxiogenic and anxiolytic effects depending on location and neuronal type (Refojo et al. 2011). Finally, in mice treated with inhaled corticosteroids without underlying lung inflammation (i.e. saline controls), a FLU-associated decrease in anxiety-related behavior was linearly associated with increased ventral hippocampal *Crhr2* expression. These results are in line with prior reports on the anxiolytic role of *Crhr2* receptors (Bale et al. 2000, Kishimoto et al. 2000), and suggest one possible functionally-signaling pathway that is altered by chronic inhaled corticosteroids during development, and one that may confer anxiety resilience. Together, the results of the current study suggest that chronically-inhaled corticosteroids can alter both glucocorticoid circulation and anxiety-like behavior and that ventral hippocampus HPA/anxiety-related genes, particularly *Crhr2*, may be an important pathway in these alterations.

#### 4.1 Conclusion

Taken together, the results of the present study provide insight into the influence of inhaled corticosteroids during development when allergic asthma is most prevalent. We did not observe dose-dependent effects of fluticasone propionate on lung inflammation in mice that were repeatedly exposed to house dust mite extract. However, we did observe that in the absence of allergic asthma, developmental exposure to fluticasone propionate resulted in health impacts like decreased weight gain, short-term elevations in baseline glucocorticoids, and decreased anxiety-like behavior. In addition, correlations between anxiety-related behavior, corticosterone, and brain gene expression indicate that anxiety-like behavior was associated with decreased circulating corticosterone levels in females and decreased ventral hippocampus *Crhr2* gene expression in males and females. Future studies can expand upon the current findings by testing effects of higher ICS doses during development, adjusting ICS dosages based on age, or including other medications in addition to fluticasone propionate. These data emphasize the importance of restricting ICS treatment to children that have a definitive allergic asthma diagnosis because chronic steroid treatment during development can alter growth, behavior, and expression of HPA-related genes in emotion-regulation areas of the brain.

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#### Abbreviations

|     |                         |
|-----|-------------------------|
| ICS | inhaled corticosteroids |
| HDM | house dust mite         |
| FLU | fluticasone propionate  |

|                |                                            |
|----------------|--------------------------------------------|
| <b>P</b>       | Postnatal day                              |
| <b>CORT</b>    | corticosterone                             |
| <b>CRH</b>     | corticotropin releasing hormone            |
| <b>HPA</b>     | hypothalamic-pituitary-adrenal axis        |
| <b>USV</b>     | ultrasonic vocalization                    |
| <b>IL-5</b>    | interleukin 5                              |
| <b>Crhr1</b>   | corticotropin releasing hormone receptor 1 |
| <b>Crhr2</b>   | corticotropin releasing hormone receptor 2 |
| <b>GR</b>      | glucocorticoid receptor                    |
| <b>EPM</b>     | elevated plus maze                         |
| <b>PFC</b>     | prefrontal cortex                          |
| <b>ANOVA</b>   | analysis of variance                       |
| <b>H&amp;E</b> | hematoxylin & eosin                        |

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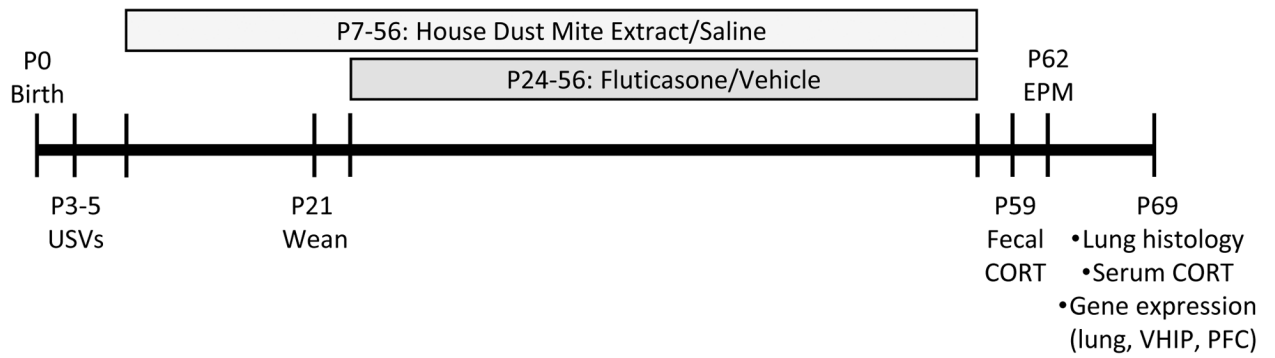


**Figure 1. Timeline for experimental study.**

Time of house dust mite extract (HDM)/saline and fluticasone (FLU)/vehicle exposure and all other samples/tests. HDM/saline exposure and FLU/vehicle exposure occurred three times/week on the same days. Abbreviations: P=postnatal day, USVs=ultrasonic vocalizations recorded, CORT=corticosteroids, EPM=elevated plus maze, VHIP=ventral hippocampus, PFC=prefrontal cortex.

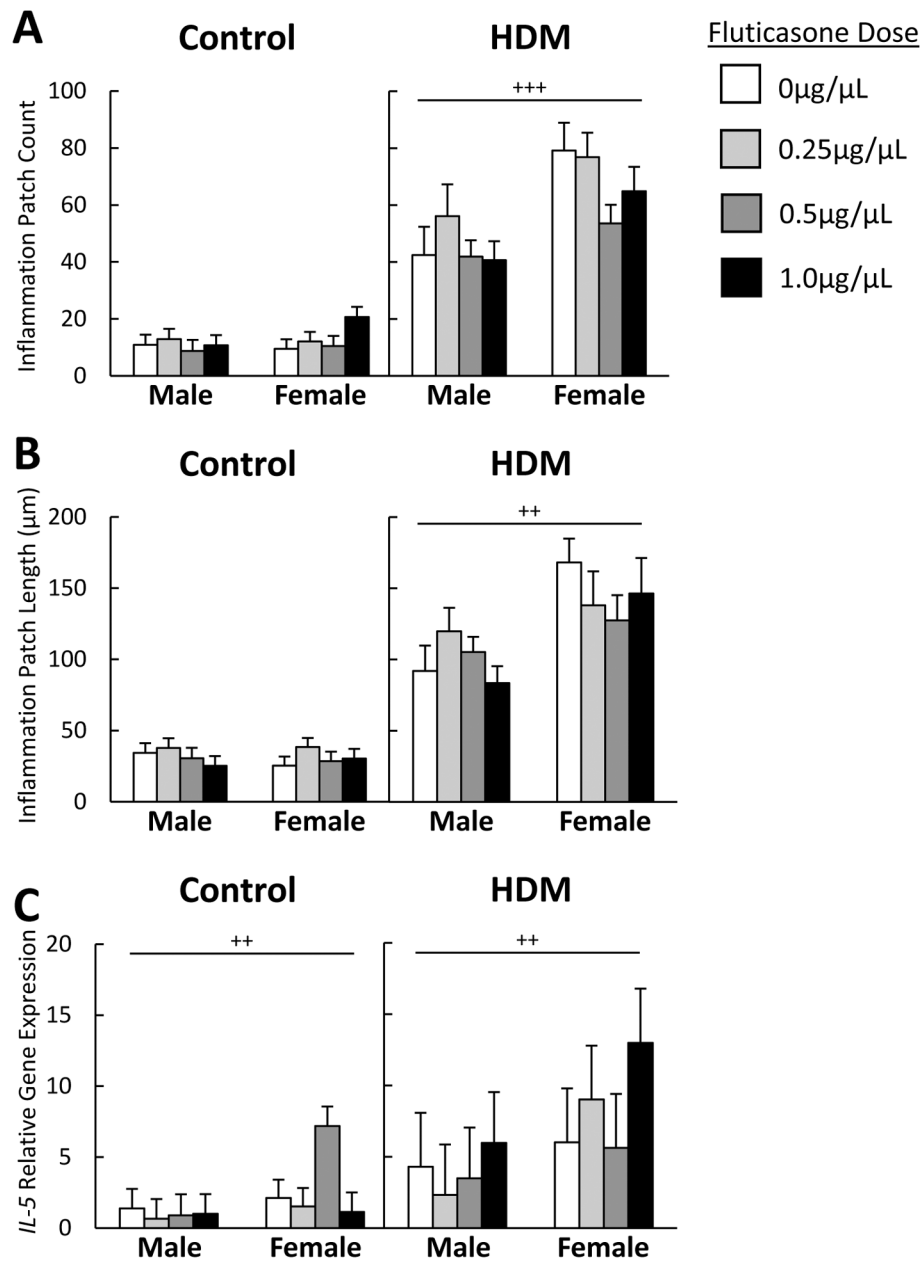


**Figure 2. Lung inflammation.**

(a, b) HDM-exposed females had more and larger lung inflammation patches than HDM-exposed males. (c) Females had greater lung *IL-5* gene expression than males. Main effects of sex F>M: ++  $p < 0.01$ , +++  $p < 0.001$ .

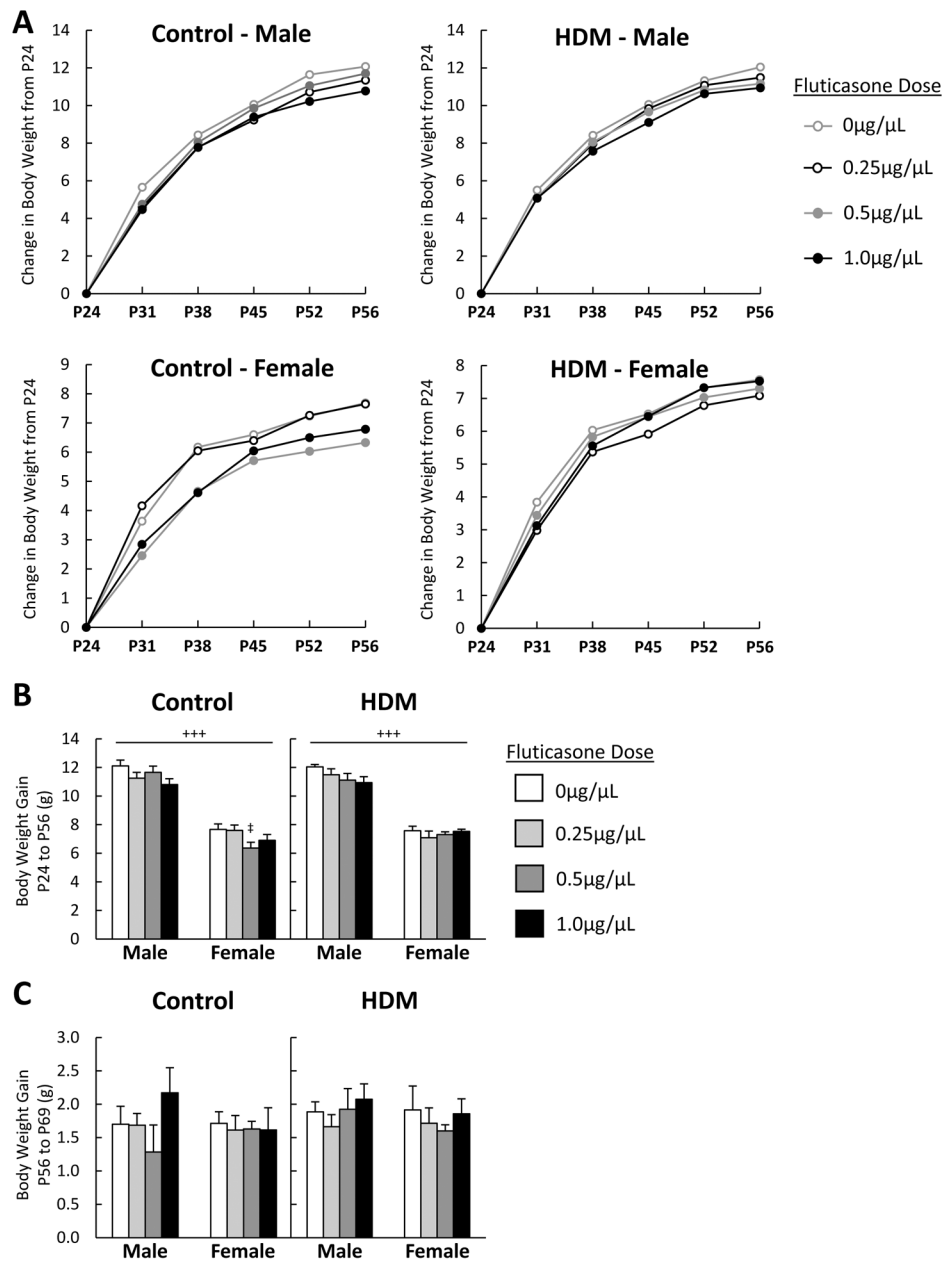


**Figure 3. Body weight gain.**

(a) Cumulative increase in body weight relative to P24 during FLU treatments for males and females in each condition. (b) In saline control females, mice treated with the moderate FLU dose (0.5µg/µL) grew less than those exposed to vehicle or low FLU dose (0, and 0.25µg/µL). During adolescence, females grew less than males. (c) During the two weeks after HDM and FLU exposure ended, there were no group differences in body weight gain. Main effects of sex  $F < M$ :  $^{+++}p < 0.001$ ; FLU dose significantly different from vehicle and low dose:  $^{\dagger}p < 0.05$ . Significant effects in repeated measures analyses are not shown on the figure but are provided in text.

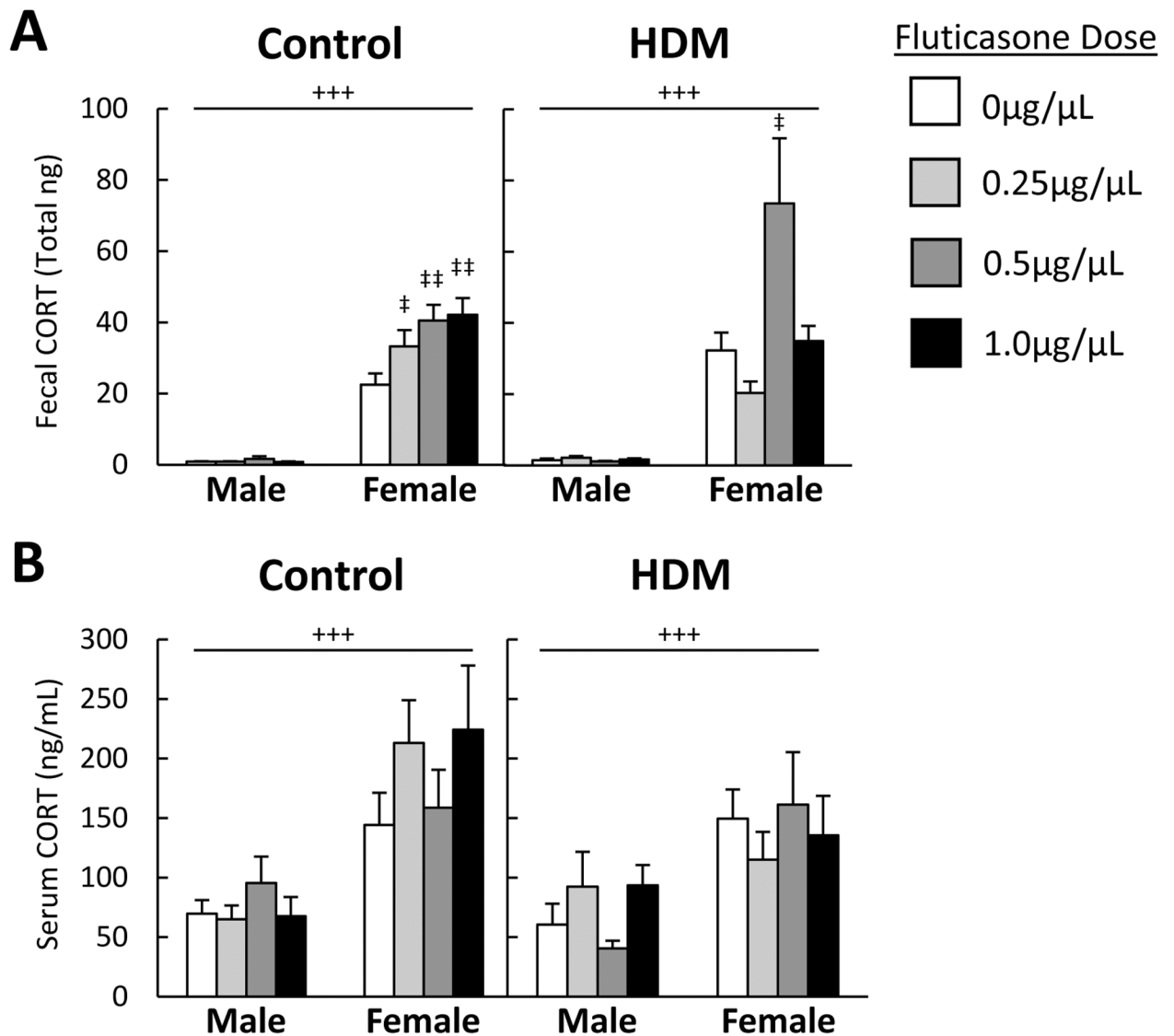


**Figure 4. Fecal and serum corticosteroids.**

(a) In saline control females, all FLU doses (0.25, 0.5, and 1.0  $\mu\text{g}/\mu\text{L}$ ) led to increased CORT excretion in feces compared to vehicle (0  $\mu\text{g}/\mu\text{L}$ ). In HDM-exposed females, only mice treated with a moderate FLU dose (0.5  $\mu\text{g}/\mu\text{L}$ ) produced more CORT than vehicle and low FLU dose groups (0  $\mu\text{g}/\mu\text{L}$ , 0.25  $\mu\text{g}/\mu\text{L}$ ). Females excreted more corticosteroid (CORT) metabolites than males. (b) Females had higher circulating CORT concentrations than males. Main effects of sex F>M: \*\*\* $p$ <0.001; FLU dose significantly different from vehicle: † $p$ <0.05, †† $p$ <0.01.

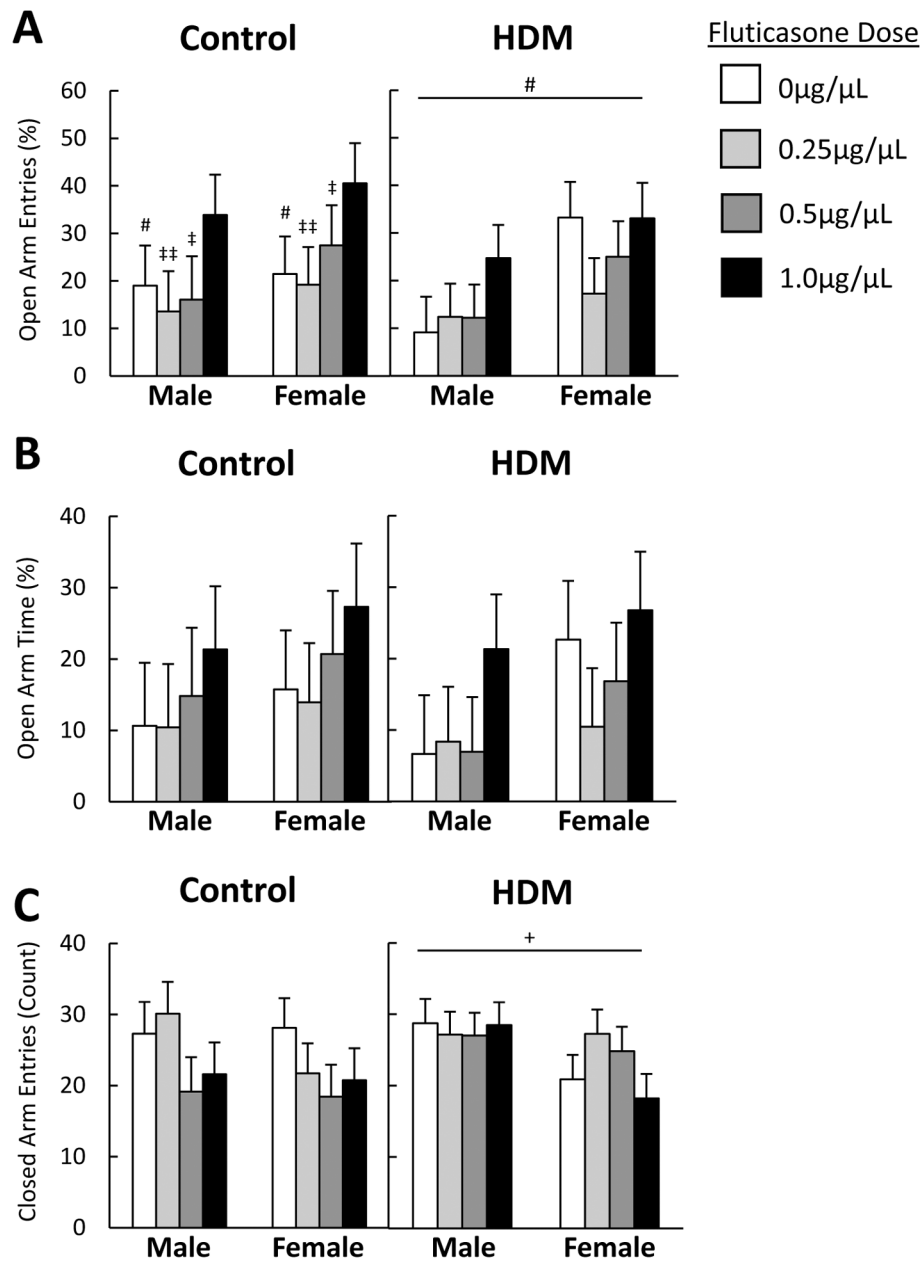


**Figure 5. Elevated plus maze behavior.**

(a) In saline control mice, those treated with the highest FLU dose (1.0  $\mu\text{g}/\mu\text{L}$ ) entered open arms of the EPM more than all other groups (0, 0.25, and 0.5  $\mu\text{g}/\mu\text{L}$ ). (b) No group differences were observed in percent open arm time. (c) HDM-exposed males entered closed arms of EPM more than females. Main effects of sex: F>M <sup>#</sup> $p < 0.10$ , F<M <sup>†</sup> $p < 0.05$ ; FLU doses significantly different from high dose: <sup>#</sup> $p < 0.10$ , <sup>†</sup> $p < 0.05$ , <sup>††</sup> $p < 0.01$ .

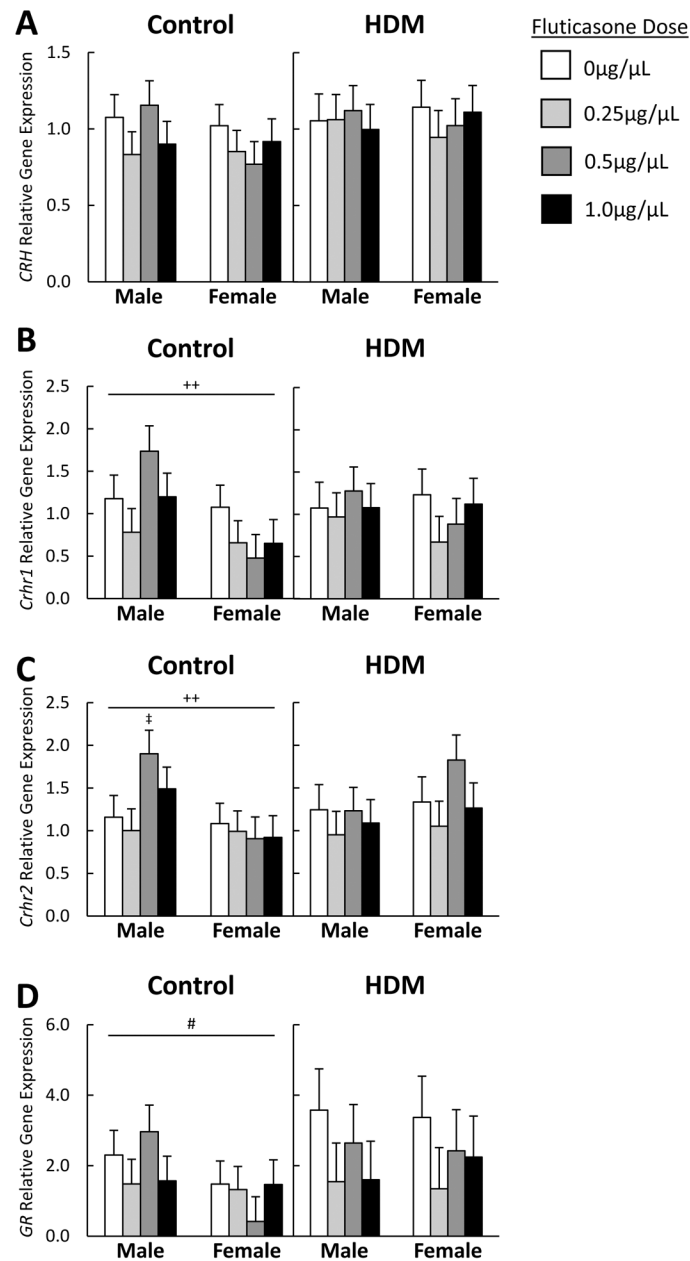


**Figure 6. Gene expression in ventral hippocampus.**

(a) No significant group differences were observed in *CRH* gene expression. (b, c, d) In saline control mice, males had greater ventral hippocampus *Crhr1*, *Crhr2*, and *GR* gene expression than females. (c) In saline control males, mice treated with moderate FLU dose (0.5 µg/µL) had greater *Crhr2* expression than vehicle and low dose males (0 and 0.25 µg/µL). Main effect of sex F<M: # $p<0.10$ , ++ $p<0.01$ ; FLU dose significantly different from vehicle and low dose: # $p<0.01$ .

**Table 1**

Allergen (HDM) effects on airway inflammation. HDM-exposed mice had greater airway inflammation, based on number of patches, length of patches, and *IL-5* gene expression, compared to saline control mice. Lung inflammation patches (counts and average length) were visualized with hematoxylin & eosin (H&E) staining. *IL-5* gene expression was quantified with real-time PCR. For parametric statistics, data were transformed (natural log) to provide a normal distribution for analysis, and *F* and *p* are reported for each variable. For clarity, untransformed means and standard error are presented in the table. Not shown in table: Inflammation measures were positively correlated with one another (patch count vs length:  $r=0.869$ ,  $df=114$ ,  $p<0.001$ ; patch count vs. *IL-5*:  $r=0.329$ ,  $df=114$ ,  $p<0.001$ ; patch length vs. *IL-5*:  $r=0.306$ ,  $df=114$ ,  $p=0.001$ ).

| Measure                               | Mean (SEM)        |                   | <i>F</i> | <i>p</i> |
|---------------------------------------|-------------------|-------------------|----------|----------|
|                                       | Saline Control    | HDM               |          |          |
| Inflammation Patch Count              | 12.027<br>(2.636) | 56.364<br>(2.591) | 158.82   | <0.001   |
| Inflammation Patch Length (μm)        | 31.185<br>(5.337) | 121.72<br>(5.246) | 99.74    | <0.001   |
| rt-PCR Gene Expression of <i>IL-5</i> | 2.54<br>(1.307)   | 6.53<br>(1.285)   | 31.071   | <0.001   |

**Table 2**

Relationship between behavior in the elevated plus maze (EPM) and serum corticosterone concentration one week later in male and female mice. EPM measures include percent open arm entries and time. Correlation analysis results ( $r$  and  $p$ ) are provided for comparisons between behavior and corticosterone. Degrees of freedom for all analyses were 56. Bold font indicates statistically significant correlation coefficients.

| Behavior in EPM      | Serum Corticosterone |       |              |       |
|----------------------|----------------------|-------|--------------|-------|
|                      | Male                 |       | Female       |       |
|                      | $r$                  | $p$   | $r$          | $p$   |
| Open Arm Entries (%) | 0.122                | 0.362 | <b>0.315</b> | 0.016 |
| Open Arm Time (%)    | 0.135                | 0.312 | <b>0.295</b> | 0.025 |

**Table 3**

Relationship between ventral hippocampus gene expression, serum corticosterone concentration, and behavior on the elevated plus maze (EPM; percent open arm entries). Correlation analysis results ( $r$  and  $p$ ) are provided for each comparison. Degrees of freedom in 'All Groups' were 56 and 56 for males and females, degrees of freedom in 'Saline-treated Control mice' were 25 and 28 for males and females respectively. Bold font indicates statistically significant correlation coefficients, marginally significant coefficients are underlined.

| ALL GROUPS                          |                         |                         |                        |                        |
|-------------------------------------|-------------------------|-------------------------|------------------------|------------------------|
| Ventral hippocampus gene expression | Serum Corticosterone    |                         | % Open Arm Entries     |                        |
|                                     | Male                    | Female                  | Male                   | Female                 |
|                                     | $r$<br>( $p$ )          | $r$<br>( $p$ )          | $r$<br>( $p$ )         | $r$<br>( $p$ )         |
| <i>CRH</i>                          | <b>-0.295</b><br>(.025) | <b>-0.323</b><br>(.013) | 0.185<br>(.164)        | 0.010<br>(.940)        |
| <i>Crhr1</i>                        | <u>-0.244</u><br>(.065) | <u>-0.229</u><br>(.083) | <b>0.300</b><br>(.022) | 0.033<br>(.807)        |
| <i>Crhr2</i>                        | -0.206<br>(.121)        | 0.002<br>(.985)         | <b>0.332</b><br>(.011) | <u>0.258</u><br>(.051) |
| <i>GR</i>                           | -0.062<br>(.642)        | <b>-0.265</b><br>(.044) | 0.154<br>(.249)        | 0.123<br>(.359)        |
| SALINE-TREATED CONTROL MICE         |                         |                         |                        |                        |
| Ventral hippocampus gene expression | Serum Corticosterone    |                         | % Open Arm Entries     |                        |
|                                     | Male                    | Female                  | Male                   | Female                 |
|                                     | $r$<br>( $p$ )          | $r$<br>( $p$ )          | $r$<br>( $p$ )         | $r$<br>( $p$ )         |
| <i>CRH</i>                          | <b>-0.446</b><br>(.020) | -0.245<br>(.193)        | 0.046<br>(.819)        | 0.087<br>(.649)        |
| <i>Crhr1</i>                        | <b>-0.412</b><br>(.033) | -0.083<br>(.664)        | 0.254<br>(.201)        | 0.180<br>(.340)        |
| <i>Crhr2</i>                        | -0.184<br>(.358)        | <u>0.307</u><br>(.098)  | <b>0.437</b><br>(.023) | <b>0.407</b><br>(.026) |
| <i>GR</i>                           | 0.037<br>(.856)         | -0.091<br>(.633)        | 0.307<br>(.120)        | 0.157<br>(.408)        |