PRE AND POSTNATAL DETERMINANTS OF AIRWAY HYPERREACTIVITY

By

Katherine M. Lebold

A DISSERTATION

Presented to the Department of Biomedical Engineering
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

May 2018

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Katherine M. Lebold

has been approved

_____

Mentor/Advisor (Dr. David Jacoby)

_____

Member (Dr. Daniel Marks)

_____

Member (Dr. Allison Fryer)

_____

Member (Dr. George Giraud)

_____

Member (Dr. Kent Thornburg)

TABLE OF CONTENTS

LIST of ABBREVIATIONS

(-)Eos: congenitally eosinophil-deficient mice

ACh: acetylcholine

ANOVA: analysis of variance

BAL: bronchoalveolar lavage fluid

BDNF: brain-derived neurotrophic factor

CC10: club cell 10kd protein

CGRP: calcitonin-gene related peptide

CHAT: choline acetyltransferase

CNS: central nervous system

E: embryonic

ELISA: enzyme-linked immunosorbent assay

F0: founding generation

F1: first generation

F2: second generation

FEV_1: volume of exhaled air in 1 second

FeNO: fractional exhaled nitric oxide

FVC: total volume of forcibly exhaled air

GDNF: glial cell line-derived neurotrophic factor

GFRα-1: GDNF family receptor alpha-1

IgE: immunoglobulin E

IgG: immunoglobulin G

IL: interleukin

IL5tg: transgenic mouse model of chronic pulmonary eosinophilic inflammation

i.p.: intraperitoneal

i.v.: intravascular

HDM: house dust mite allergen

LPS: lipopolysaccaride

P: postnatal

PBS: phosphate buffered saline

NANC: nonadrenergic, noncholinergic

NGF: nerve growth factor

NK1: neurokinin-1 receptor

NK2: neurokinin-2 receptor

nNOS: neuronal nitric oxide synthase

PGP9.5: protein gene product 9.5 (a.k.a. ubiquitin carboxyl-terminal hydrolase isozyme L1 or UCHL1).

SEM: standard error of the mean

SubP: substance P

TBS: tris buffered saline

TNFα: tumor necrosis factor alpha

TrkA: tropomyosin receptor kinase A

TrkB: tropomyosin receptor kinase B

TH: tyrosine hydroxylase

VEH: vehicle

WT: wild-type C57Bl/6 mice

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I am incredibly thankful for the support of my mentors, fellow lab members, friends and family. This short space is insufficient to express my gratitude for you all, but know I will forever be indebted to you for your help, guidance, love and support during my training.

To David, thank you for teaching me how to think critically about my data and creatively about science. You've taught me how to ask important questions and navigate failure while remaining positive. You serve as an example of how to successfully straddle the worlds of science and medicine and inspire me to work harder to achieve the same. Your mentorship is invaluable.

To Allison, thank you for teaching me how to be a diplomat and thrive as an assertive female scientist. You've taught me how to effectively communicate my science and promote my work/self on national and international levels. Thank you for supporting and advocating for me in all my professional pursuits, both in and outside the lab.

To my fellow lab members, you're the reason I joined the lab. Thank you for listening, giving good advice, helping me when I failed, having an open door that I could constantly run in and out of, and helping me manage a large amount of data. You're like a second family.

To my committee, thank you for your time. It is the most valuable thing you have and you gave of it and your advice freely to teach me how to be a scientist. You helped me design and think about experiments, supported me when I struggled with my career path, and enthusiastically advocated for me when I figured it out.

To the broader MD/PhD program, you're my people. Thank you for building a community where we can all succeed and support each other.

To my friends, thank you for the love, free therapy, runs, countless dinners and happy hours, reality checks, late night dancing and rapping, vacations, and so much more. I love living this life with all of you in it.

To my family, thanks for putting up with me on this long journey. Thank you for understanding my absenteeism and believing in my dream as much as I do. To my husband, you make the world revolve and keep me fed. You make sure our life is tended to while I pursue my career. I couldn't do all we do without you.

ABSTRACT

Asthma is characterized by airway hyperreactivity, an abnormal tendency for airways to constrict. Maternal asthma increases the risk of childhood asthma more than paternal disease, suggesting intrauterine exposures contribute to airway hyperreactivity. As airway hyperreactivity seldom returns to the normal range despite intensive treatment, this suggests that structural alterations with *in utero* origins impair lung function lifelong.

Airway innervation mediates airway hyperreactivity. Afferent epithelial sensory nerves respond to inhaled stimuli and trigger activation of efferent parasympathetic nerves. These efferent nerves activate parasympathetic ganglia in the airways, releasing acetylcholine, which contracts airway smooth muscle. Blocking this reflex with muscarinic antagonists improves lung function, demonstrating the importance of airway innervation to asthma pathogenesis.

I hypothesized that airway hyperreactivity in offspring born to mothers with asthma is established during development and results from increased lung sensory innervation. Here I show that pregnant mice exposed to house dust mite, a model of asthma, give birth to offspring with markedly increased lung sensory innervation, long lasting increases in epithelial neurotrophins, and airway hyperreactivity without changes in type 2 cytokines in the offspring.

Eosinophils, a white blood cell, are recruited to the lungs after allergen exposure where they interact with nerves to increase airway hyperreactivity. To test if increased offspring airway innervation results from maternal interleukin-5, which promotes eosinophil hematopoiesis, and fetal eosinophilia, I measured airway innervation in wildtype offspring born to IL-5 transgenic mothers. Pregnant mice with elevated IL-5 gave birth to offspring with airway hyperreactivity, increased airway innervation, and increased epithelial neurotrophin expression that was dependent on fetal eosinophilia. The fetal eosinophilia was due to maternal IL-5 crossing the placenta.

Neither offspring of house dust mite exposed mothers nor offspring of IL-5 transgenic mothers have airway inflammation as adults, demonstrating that persistent airway hyperreactivity does not depend on continual inflammation. However, when airway inflammation was induced in these animals, the combination of inflammation with increased innervation caused severe, lethal hyperreactivity.

Humans and mice are born with immature lungs with substantial structural development continuing after birth. Airway innervation continues to develop after birth and environmental insults during this critical period permanently increase airway innervation to promote airway hyperreactivity. Here I also show that airway parasympathetic ganglia continue to develop after birth and contain rare populations of neurons that express substance P, nitric oxide synthase, and

tyrosine hydroxlase. Increasing eosinophil influx into the lungs after birth arrests parasympathetic ganglia development, resulting in fewer ganglia, less heterogeneous neurotransmitter expression, and associated airway hyperreactivity. Deletion of eosinophils prevents airway hyperreactivity, and these animals have increased numbers of parasympathetic ganglia and increased neurotransmitter heterogeneity.

These results demonstrate that airway hyperreactivity results from aberrant nerve development, occurring *in utero* for afferent nerves and after birth for efferent nerves. Perturbing the maternal immune system directly alters sensory innervation of developing lungs and thus fetal exposure to maternal asthma or increased IL-5 contributes to developmental origins of airway hyperreactivity. During the postnatal period, eosinophils shape parasympathetic ganglia development and environmental insults that increase eosinophils during this critical window may permanently alter airway innervation.

CHAPTER 1. Introduction

A. Asthma Overview

A1. Definitions

Asthma is a chronic respiratory disease of global importance. Upwards of 300 million people have asthma and disease occurs irrespective of country of origin, ethnic background, age or gender. Asthma prevalence is on the rise worldwide driven by increases in urbanization, air pollution, and allergic sensitization[1]. Patients with asthma present with respiratory symptoms such as shortness of breath, wheezing, chest tightness and cough that vary in intensity, duration, and triggers. Diagnostic criteria for asthma include the presence of respiratory symptoms with documented airflow obstruction that is reversible or excessively variable[2].

By definition, asthma is a heterogeneous disease with divergent underlying mechanisms and subsequent response to therapy. In attempts to improve treatment, diagnostic criteria, along with genetic and environmental factors, were used to define early clinical asthma phenotypes such as “early-onset” vs. “late-onset” asthma. As research technologies advanced, phenotypes evolved to incorporate the following pathologic features of asthma: *airway inflammation, remodeling, and hyperreactivity* where the airways constrict excessively to nonspecific stimuli. The combination of observable clinical characteristics with pathologic, measurable features resulted in phenotypes such as “early-onset, eosinophilic allergic asthma”. The need to identify pathobiologic mechanisms

with clinical utility led to the creation of asthma endotypes, which marries molecular biomarkers with clinical characteristics, lung physiology, histopathology, and treatment response[3] in hope of creating improved, personalized treatment options. Asthma remains an umbrella diagnosis clinically, however the definitions and identification of asthma subtypes are rapidly evolving.

A2. Physiology: Airway Hyperreactivity

Airway reactivity describes how the airways constrict in response to direct or indirect stimuli. In clinical settings, airway responsiveness is assessed using spirometry to measure exhaled airflow before and after exposure to an inhaled contractile agonist, i.e. inhaled methacholine, histamine, or mannitol. Airways constrict after step-wise increases in contractile agonist concentration, leading to a fall in exhaled air. Reduced airflow is detected as a decrease in the volume of exhaled air in 1 second (FEV1). In patients with asthma, the concentration of contractile agonist that causes a 20% reduction in FEV_1 is less than age- and gender-matched ranges for individuals without asthma[4] and thus considered a marker of airway hyperreactivity.

Spirometry also measures the total volume of forcibly exhaled air, termed the forced vital capacity (FVC). When airflow obstruction is present at baseline, irrespective of inhaling a contractile agonist, the FEV_1 is reduced out of proportion to the FVC. Thus, a FEV_1/FVC ratio of 0.7 (70%) indicates obstructive

lung physiology. A bronchodilator can be inhaled (i.e. albuterol) after baseline spirometry is measured to test if airflow obstruction is reversible. When the FEV_1 measured after inhalation of a bronchodilator increases >12% and 200 ml as compared to the pre-bronchodilator FEV_1[2], subjects are considered to have reversible airflow obstruction. Reversibility of airflow obstruction is a hallmark of asthmatic airway physiology.

Two components of airway hyperreactivity are important in asthma: the first is a baseline, persistent responsiveness that reflects abnormal airway structures and, with current treatments, is irreversible. The second component is acute hyperreactivity due to airway inflammation[4] that is suppressed by inhaled steroids, but seldom to the range of individuals without asthma[5]. Airway hyperreactivity occurs in the absence[6] or after the resolution of airway inflammation[7,8], again demonstrating a baseline component of airway hyperreactivity. Thus, novel therapeutics designed to reduce hyperreactivity will need to address both persistent and inflammation-induced hyperreactivity.

A3. Asthma Phenotypes and Endotypes

Asthma phenotypes and endotypes identify patients who will benefit from specific therapies and offer insight into disease severity and progression. Asthma phenotypes use observable clinical characteristics (ex: severity, age of onset, etc.) and environmental factors (ex: symptom triggers) to define patients, while

endotypes are subcategories within phenotypes that incorporate specific biomarkers and treatment response.

A3.1 Overview of "Type-2 High" Asthma

Most asthma biomarkers have been identified in patients with severe disease and focus on airway inflammation. Immune responses to environmental exposures have classically been defined by polarization of and cytokine secretion from T lymphocytes and innate lymphoid cells. Type 1 lymphocytes secrete interferon-gamma, interleukin-2, and tumor necrosis factor-beta. Activated Type 2 lymphocytes secrete interleukin (IL)-4, IL-5 and IL-13 in individuals with atopic asthma[9] and this is broadly termed "type 2" inflammation. Interstitial innate lymphoid cells are enriched at mucosal sites and follow the same cytokine and inflammatory subtyping as T lymphocytes. Thus innate lymphoid cells that secrete interferon-gamma are considered type 1 while type 2 innate lymphoid cells secrete IL-5 and IL-13.

A primary method to distinguish patients, therefore, is degree of cytokine expression, i.e. "type-2 high" versus "type-2 low" asthma[10]. Many "type-2 high" patients also have elevated eosinophils, prominent airway remodeling, and airway hyperreactivity[10]. Allergen sensitization is common in patients with "type-2 high" asthma, however allergic asthma is defined by reactivity to allergens and production of immunoglobulinE (IgE)[11], and patients with non-atopic asthma can be either "type-2 high"[12] or "type-2 low". Few reliable, targetable biomarkers have

been identified for “type-2 low” asthma, leading to a deficit of treatments options for these patients. In contrast, “type-2 high” asthma has targetable biomarkers (IL-5, IL-4, and IL-13) by definition, resulting in a rapid expansion of therapeutic options and additional biomarkers as detailed below.

A3.2 Type 2 Cytokines

IL-5 stimulates eosinophil hematopoiesis, survival and activation and is secreted by type 2 innate lymphoid cells and CD4+ T lymphocytes. Airway epithelial cells secrete alarmin cytokines (IL-25, IL-33 and thymic stromal lymphopoetin) after exposure to allergen or other damaging insults that signal to lung interstitial type 2 innate lymphoid cells to release IL-5[13]. Dendritic cells process allergens, traffic to draining lymph nodes, and after antigen presentation, induce CD4+ T lymphocytes polarization and subsequent secretion of IL-5[14]. Genetic deletion or pharmacologic neutralization of IL-5 in animal models of asthma reduces airway inflammation, remodeling and hyperreactivity[15-17] and led to the development of mepolizumab, reszilumab and benralizumab.

IL-4 and IL-13 share a common receptor subunit and thus have several redundant, overlapping roles as well as unique functions in asthma pathogenesis. IL-4 is secreted predominantly from CD4+ T lymphocytes and amplifies type 2 polarization of the immune response, thus enhancing airway eosinophilic inflammation and B cell derived IgE[18]. Consequently, genetic deletion or pharmacologic neutralization of IL-4 in animal models of asthma

reduces airway inflammation[19,20] and hyperreactivity[21]. Type 2 innate lymphoid cells secrete IL-13, but not IL-4, when exposed to the airway epithelial alarmin cytokines (IL-25, IL-33 and thymic stromal lymphopoetin)[13]. Antigen presentation by dendritic cells and polarization of CD4+ T lymphocytes also leads to secretion of IL-13[14]. Independent of allergen exposure, IL-13 can induce eosinophilic lung inflammation, IgE synthesis, airway hyperreactivity, and mucus production[22]. After allergen exposure, blocking IL-13 can reduce allergen-induced airway hyperreactivity, mucus production and inflammation[22,23]. The combined functions of IL-4 and IL-13 led to the development of dupulimab, a receptor antagonist that blocks signaling from both IL-4 and IL-13, as well as lebrikizumab that selectively neutralizes IL-13.

A3.3 Eosinophils

Eosinophils are elevated in many patients with asthma and correlate with disease severity[24,25], exacerbation frequency[26], and lung function[27]. Like many immune cells, subsets of eosinophils exist. Regulatory eosinophils reside in lungs under normal conditions, but are IL-5 independent, don't expand after allergen exposure, and express genes important for tissue homeostasis and suppression of inflammation[28]. Allergen exposure robustly increases inflammatory eosinophil recruitment to lungs. Inflammatory eosinophils are bone marrow derived[29,30], suppressed by steroids[31], IL-5-dependent, and express pro-inflammatory genes[28]. Anti-IL-5 therapies do not completely suppress airway eosinophils in humans[32], and treatment differentially affects surface marker expression on lung

versus newly recruited, circulating eosinophils[33], suggesting multiple eosinophil populations also exist in humans.

Eosinophils are not required for allergen sensitization[34], but genetic deletion of eosinophils protects against airway hyperreactivity and remodeling after allergen exposure[35]. Adoptive transfer of eosinophils restores reduced airway hyperreactivity in allergen-exposed IL-5 knockout mice[36] and recruitment of eosinophils to the lung using IL-5 causes airway hyperreactivity and remodeling without allergen exposure[37], demonstrating that eosinophils are both necessary and sufficient to cause airway hyperreactivity. Eosinophils accumulate and degranulate in lungs after allergen exposure[38], releasing a host of granule proteins (major basic protein and eosinophil peroxidase), chemokines, cytokines, and growth factors[39,40]. How and under what conditions individual eosinophil-derived mediators promote airway hyperreactivity is an area of active research. Eosinophil-specific deletion of IL-13, but not major basic protein or eosinophil peroxidase[41], inhibits eosinophil-induced airway hyperreactivity in the absence of allergen in mice. The role of major basic protein after allergen exposure varies by species, with potentiation of airway hyperreactivity in guinea pigs[42] and no effect on allergen-induced airway hyperreactivity in mice[43]. The recent development of selective knockout technology[44] will further clarify the role of specific eosinophil-derived products in airway hyperreactivity.

A3.4 Additional Type-2 Biomarkers.

Additional biomarkers used to define asthma endotypes include IgE, periostin and fractional exhaled nitric oxide. IgE is an allergen-specific antibody secreted by B cells that induces mast-cell degranulation and identifies allergic or atopic asthma phenotypes. Periostin is an extracellular matrix protein that is increased by IL-13[45,46] and measurable in peripheral blood. Serum periostin correlates with sputum eosinophilia[47,48] and is used to phenotype patients with type 2 inflammation. Finally, epithelial cells release nitric oxide in response to inflammatory insults and thus increase the fraction of exhaled nitric oxide; it is generally considered another marker of type 2 immune responses.

A4. Treatments

Asthma treatments aim to reduce exacerbations, improve lung function, and relieve symptoms by suppressing airway inflammation (inhaled steroids) and reversing bronchoconstriction (β_2 adrenergic receptor agonists or muscarinic receptor antagonists). Currently, there are no curative options or medications that completely reverse airway remodeling and airway hyperreactivity. Inhaled steroids reduce symptoms and improve lung function, however, many patients remain symptomatic and require additional treatment. The introduction of asthma endotyping based on molecular biomarkers has led to the rapid development of novel biologic drugs including omalizumab, mepolizumab, reszilumab, benralizumab, lebrikizumab and dupulimab.

A4.1 IgE Targeted Therapy

Omalizumab binds to and neutralizes IgE and was the first biologic therapy brought to market for asthma. Omalizumab reduces exacerbations[49,50] and improves lung function[51] of patients with asthma. Omalizumab is recommended as add-on therapy for patients with poorly controlled disease despite receiving inhaled steroids and long-acting β_2 adrenergic receptor agonists.

A4.2 IL-5 Targeted Therapies

Mepolizumab is a monoclonal antibody that neutralizes IL-5 and reduces blood and sputum eosinophils[52]. In clinical trials, mepolizumab improved asthma symptoms, reduced exacerbations, and decreased steroid use in patients with severe asthma[53-55]. Patients with high peripheral blood eosinophil counts benefited most from mepolizumab therapy[56], thus it is approved for patients with poorly controlled disease despite receiving inhaled steroids and long-acting β_2 adrenergic receptor agonists and who have an eosinophilic phenotype. A similar drug, reslizumab, also neutralizes IL-5 and decreases sputum eosinophils[57], resulting in fewer exacerbations[57,58] and modestly improves lung function in patients with an eosinophilic phenotype[59]. Benralizumb targets the IL-5 receptor and reduces airway sputum, submucosal, and blood eosinophils[60]. In clinical trials, benralizumab reduced exacerbation frequency[61,62] and steroid use[63] and recently received approval for use in individuals with severe, eosinophilic asthma. None of the anti-IL-5 drugs significantly improve lung function, but since asthma

exacerbations accelerate lung function decline[64], it is hopeful these medications will preserve lung function in patients with asthma.

A4.3 IL-4 and IL-13 Targeted Therapies

Two biologics are currently under development that target IL-4 and IL-13 pathways. Lebrikizumab neutralizes IL-13 and modestly improves lung function in patients with periostin high phenotypes[65,66]. However, lebrikizumab does not consistently reduce exacerbations and improvements in lung function varied across trials for specific biomarker-defined groups[67]. Dupilumab is a monoclonal antibody against the IL-4α receptor unit and thus blocks both IL-4 and IL-13 signaling. Dupilumab reduces exacerbations and improves lung function[68]. Clinical trials for both lebrikizumab and dupilumab are ongoing.

B. Childhood Asthma

B1. Risk Factors

Asthma often begins in childhood with over half of adult patients reporting symptoms during adolescence[69]. Asthma decreases quality of life[70] and imposes significant burdens during childhood while profoundly impairing lifelong health. Lung function trajectories from childhood through young adulthood are abnormal in 75% of individuals with asthma[71]. Pediatric lung function strongly predicts adult lung function[72] and asthma risk[73-75], emphasizing the need for management and prevention of childhood asthma to optimize lifelong lung health.

Invasiveness and requisite cooperation for pulmonary function tests makes the diagnosis of asthma in young children difficult. Adult markers of disease, namely symptomatic wheeze, and empiric treatment response are surrogates for assessing childhood asthma. Episodic, early-onset wheeze is common in children and often due to recurrent respiratory viral infections. Wheeze resolves in most children before puberty[76,77] and has little impact on long-term lung function in non-atopic children[78]. Thus, persistent wheeze, independent of respiratory infection, together with concomitant asthma risk factors and positive treatment response are suggestive of asthma in young children. Important risk factors for childhood asthma include parent atopy and smoking, male sex, low birth weight, lower socioeconomic status[79], early life respiratory viral infections[80] and prematurity[81]. Early sensitization to house dust mites, airway hyperreactivity, and female sex predict wheeze persistence[82].

B2. Role of Maternal Asthma

B2.1 Overview

Parental asthma increases the risk of their child developing asthma[83,84]. Genetic inheritance of risk alleles and shared environments explain only a part of this risk. Maternal asthma imposes a greater risk for childhood asthma than paternal asthma[85], suggesting a role for prenatal exposures in asthma susceptibility. Mothers with severe, uncontrolled asthma give birth to children with a greater risk of developing asthma than children from mothers with mild, controlled asthma[86,87], raising the question whether maternal inflammation affects fetal

airway development. Woman with asthma who are intensively managed during pregnancy using fraction of exhaled nitric oxide, a marker of type-2 inflammation, to guide therapy give birth to infants with reduced risk of respiratory illness[88] and asthma[89], compared to children born to mothers with symptom-only based care, demonstrating that targeting maternal inflammation can reduce asthma risk in children.

Maternal asthma may influence childhood asthma risk through multiple pathways and at various time points. Airway reactivity varies at birth in healthy newborn animals[90], suggesting *in utero* programming of baseline airway reactivity. Maternal atopy and asthma associate with impaired infant lung function[91] and airway hyperreactivity[92], independent of allergen sensitization at birth. This neonatal airway hyperreactivity increases risk of asthma in adolescence[93,94], demonstrating prenatal exposure to maternal asthma changes lung development with functional, long-term consequences. In the postnatal environment, airway hyperreactivity at baseline in children exposed to maternal asthma is augmented by allergen sensitization. Airway hyperreactivity correlates with IgE levels in children[95] and allergen sensitization predicts the persistence of wheeze[82]. Allergen sensitization requires immune cell activity. Infants born to parents with asthma have heightened mononuclear proliferative responses to allergens at birth[96,97], suggesting *in utero* exposure to maternal asthma reprograms inflammatory cells. Genetic inheritance does not entirely explain altered immune responses at birth as infants born to mothers with asthma who are exposed to

cat dander develop wheeze, while children born to fathers with asthma or mothers without asthma are protected[98].

B2.2 Structural Remodeling and Epigenetics

The persistent effect of *in utero* exposures on postnatal lung function, in the absence of genomic change, requires either structural change to developing tissues or genome epigenetic modification. Impaired lung function and airway hyperreactivity at birth suggest structural changes occur during development when the fetus is exposed to maternal asthma. Bronchial biopsies from young children at risk for asthma demonstrate epithelial basement membrane thickening[99] and airway epithelium and sputum from children with asthma contain more pro-remodeling factors than epithelium or sputum from children without asthma[100,101]. However, the influence of maternal asthma on structural remodeling in the offspring lung has not examined.

Epigenetic modifications include methylation, acetylation, phosphorylation, ubiquitylation, or sumolyation of DNA or histones, complexes that bundle DNA and control transcription access. DNA methylation is the best understood, where promoter hypomethylation increases expression and hypermethylation decreases expression of a gene. DNA methylation is a dynamic process that is heritable but also changes throughout life in response to environmental exposures. DNA methylation is a powerful mechanism to transmit information to successive generations, resulting in rapid biologic adaptation to environmental exposures.

However, such a dynamic process makes it difficult to test how methylation of specific genes contributes to asthma. Even more difficult is to identify cell types with methylation differences that promote specific pathologic features of asthma.

Despite these limitations, differential methylation has been found in newborns who develop asthma during adolescence compared with newborns who never develop asthma[102,103]. Additional studies identified epigenetic modifications in children who already have asthma compared to children without asthma[104-108]. However, such changes are usually not present at birth or predictive of disease[105,109], demonstrating the influence of postnatal exposures[109], allergen sensitization and exposure[110-112], and inflammatory cytokines[113] in shaping DNA methylation. Clusters of differentially methylation genes associate with clinical phenotypes[113,114], however it is unclear how methylation of a specific gene promoter modifies expression that subsequently causes a pathologic feature of asthma. How maternal asthma modifies this complex system is also not clear. Infants exposed to maternal asthma have differentially methylated sequences in whole blood compared to infants not exposed to maternal asthma[115], but with unknown significance. Maternal asthma may modify how gene methylation influences disease risk, such that methylation of specific genes is important for asthma risk only in children born to mothers with asthma[103]. Similarly, differential methylation at birth may alter subsequent methylation changes in response to allergen sensitization[116]. Asthma risk and DNA methylation changes also persist across generations, with documented inheritance in offspring from grandmothers

exposed to environmental insults[117-119], further confounding the question whether observed changes in children are due to maternal or grandmaternal factors.

B2.3 Maternal Inflammatory Cytokines

Transmission of asthma risk can occur independent of specific allergens in mice[120]. Broadly targeting maternal inflammation reduces childhood asthma risk in humans[89], suggesting maternal inflammatory responses to allergen exposure, as opposed to the allergen itself, affect the developing fetus. Fetal sensitization to allergens *in utero* is possible[121], and both allergen[122] and allergen-specific immunoglobulins[123] are detectable in human cord blood. However, lymphoproliferative responses to allergen at birth are not predictive of sensitization and immune responses in adolescence[124], thus making fetal responses directly to allergens an unlikely mechanism of enhanced asthma risk in offspring exposed to maternal asthma.

Allergen exposure in pregnant mice augments offspring airway inflammation and airway hyperreactivity after allergen exposure later in life[120,125]. Enhanced offspring inflammation can be suppressed by exposing mothers to interferon-Ɣ[126] or lipopolysaccharide[127], which increases secretion of many cytokines including tumor necrosis factor-α (TNFα), IL-6, and IL-1β. Blocking maternal IL-4 before maternal allergen exposure also reduces offspring airway hyperreactivity and inflammation[120]. The balance between IL-4 and interferon-Ɣ in human pregnancies is associated with childhood atopy[128], and maternal serum IL-5

correlates with infant asthma-like symptoms[129], suggesting excessive maternal type-2 inflammation or an imbalance between type-2 and type-1 inflammation may increase childhood asthma risk. Maternal cytokines could affect a developing fetus directly, by passing through the placenta, or by modulating placental function. Cytokine passage across placentas is variable[130-132], both among human studies and possibly between species due to different placental structures[133,134]. While blocking maternal IL-4 reduces offspring airway inflammation in mice[120], IL-4 and IL-13 do not cross the murine placenta[135], suggesting an indirect role for IL-4 on offspring asthma risk. Placenta also secrete cytokines[136-139] and proinflammatory cytokines expression increases after lipopolysaccharide exposure[140]. Placentas from woman with asthma have different cytokine expression[141] than placentas from woman without asthma, thus the source of inflammatory cytokines may either be systemic maternal circulation or local production by placenta.

B2.4 Fetal Growth and Placental Morphology

The *in utero* environment shapes fetal growth, which impacts the risk for chronic disease later in life[142]. Low birth weight is associated with reduced adult lung function[143] and an increased risk of asthma[144], demonstrating a correlation between intrauterine growth and asthma risk. Birth weight is positively correlated with placental size and placental dimensions also predict the risk of adult chronic disease[145], including asthma[144]. Maternal asthma restricts fetal growth[146] and low birth weight is more common in children born to women with asthma compared to

children born to woman without asthma[147]. Placentas from woman with asthma are also morphologically different[148] compared to placentas from woman without asthma. Together these data suggest that impaired placentation and restricted fetal growth contribute to increased asthma risk in children born to mothers with asthma.

C. Airway Nervous System

C1. Nerve Anatomy Overview

Airway innervation is structurally and functionally complex, involving afferent and efferent pathways, regulation by the central nervous system, and coordination of sensory, parasympathetic, sympathetic, and nonadrenergic noncholinergic (NANC) nerve subtypes. The following section describes adult lung innervation, excluding discussion of the central nervous system. A subsequent section will highlight nerve development.

C1.1 Sensory Afferent Nerve Populations

Three types of pulmonary sensory afferents exist and are defined by their electrophysiologic properties: rapidly adapting stretch receptors, slowly adapting stretch receptors, and C-fibers. Pulmonary afferents reside collectively in the vagus nerve and have cell bodies located in the vagal ganglion at the base of the skull.

Rapidly adapting stretch receptors are myelinated nerve fibers that comprise half of lung afferents, innervate tracheal and bronchial walls, and respond rapidly with low thresholds to mechanical and hyperosmotic stimuli[149]. Cell bodies of these nerves are located in nodose ganglia[149], which together with jugular ganglia, constitute the vagal ganglia.

Slowly adapting stretch receptors are myelinated nerve fibers that comprise 25% of lung afferents[149] and innervate large and small airways[150,151]. Slowly adapting stretch receptors respond slowly with high thresholds to mechanical stimuli and also depolarize when exposed to chemical stimuli[149]. Cell bodies of these nerves are large in diameter (>20μm) and located in jugular ganglia[149].

Remaining lung afferents are nociceptive, C-fibers that are unmyelinated, respond vigorously to chemical stimuli[149] and densely innervate airway epithelium[152] and other lung compartments. Cell bodies of these nerves are small in diameter (<20μm) and located in jugular ganglia[149]. Thoracic dorsal root ganglia C-fibers also innervate the distal airways, but contribute less to pulmonary physiology compared with vagal C-fibers.

C1.2 Efferent Nerve Populations

Efferent pulmonary nerves are largely defined by neurotransmitter content and include parasympathetic, NANC and sympathetic nerves.

Parasympathetic nerves control airway tone at baseline[153] and in response to afferent nerve activation[154]. Efferent parasympathetic nerves include preganglionic nerve fibers arising from the brainstem that course to the airway in the vagus nerve, clusters of ganglia embedded in trachea and bronchi, and postganglionic nerve fibers that innervate smooth muscle and submucosal glands. Individual neurons within airway ganglia have distinct electrical properties[155,156] and synapse on other neurons[156], suggesting heterogeneity within and communication between ganglia. Interganglionic communication likely gives rise to integration and filtering of preganglionic signals observed in airway parasympathetic ganglia[155,157]. Activation of efferent nerves results in acetylcholine release, which binds to M_3-muscarinic receptors and causes smooth muscle contraction[158]. M_2-muscarinic receptors located on postganglionic nerves[159,160] inhibit further acetylcholine release. Genetic deletion[158] or pharmacologic inhibition[160] of M_2-muscarinic receptors potentiates vagally mediated bronchoconstriction, demonstrating the importance of feedback inhibition of acetylcholine *in vivo*.

NANC nerves are classified as either excitatory or inhibitory depending on neurotransmitter expression. Nitric oxide and vasoactive intestinal peptide identify inhibitory NANC nerves that cause bronchodilation. NANC nerve cell bodies are predominantly located on the esophagus[161,162] and send projections to the airway, although some neurons in vagal ganglia[163] and

airway parasympathetic ganglia[162,164] express nitric oxide synthesizing enzymes as well. Depolarization of afferent sensory nerves by electrical field stimulation or chemical stimuli causes local reflex activation of NANC nerves to relax contracted smooth muscle[165].

Expression of substance P identifies excitatory NANC nerves that cause bronchoconstriction, increase plasma extravasation, and enhance inflammatory cell recruitment. Most excitatory NANC nerves are actually sensory afferents with cell bodies located in vagal ganglia. However, activation of sensory afferents produces local axon reflexes[165,166], releasing excitatory neurotransmitters in the periphery[167]. Excitatory NANC nerves thus have both afferent and efferent functions. The specific function of substance P is detailed in subsequent sections.

Sympathetic nerves secrete norepinephrine and have cell bodies located in superior cervical, stellate, and paravertebral ganglia. Most sympathetic nerves supplying the lung are largely restricted to pulmonary vasculature[168] with minimal smooth muscle innervation[169]. However, smooth muscle robustly expresses β_2-adrenergic receptors and relaxation occurs when exposed to norepinephrine[170,171] or β_2-adrenergic agonists[172].

C2. Asthma

C2.1 Reflex Bronchoconstriction

Smooth muscle contraction constricts the airways and occurs after direct exposure to contractile agonists, or indirectly, due to nerve activation and subsequent release of acetylcholine. Inhaled or systemic agents can activate efferent parasympathetic ganglia directly, leading to acetylcholine release and bronchoconstriction, while reflex bronchoconstriction involves coordinated activation of both afferent and efferent pathways. In reflex bronchoconstriction (Figure 1), afferent epithelial sensory nerves with cell bodies located in the nodose-jugular ganglia of the vagus respond to inhaled stimuli, synapse on central nervous system circuits, and subsequently trigger activation of efferent parasympathetic nerves. These efferent nerves activate parasympathetic ganglia embedded within the lungs, releasing acetylcholine from postganglionic parasympathetic nerves which contracts airway smooth muscle. Activation of epithelial afferents also stimulates neighboring sensory nerves to release neurotransmitters without central nervous system involvement.

Airway responses to methacholine[173,174], histamine[175,176], bradykinin[177], capsaicin[178], serotonin[179] and allergen[180] all involve reflex bronchoconstriction which is blocked by muscarinic antagonists (ipratropium[178] or atropine[176,177,180]), cutting the vagus nerves[174-177,180], or depleting afferent neurotransmitters[181]. Reflex bronchoconstriction is heightened in individuals with asthma[154] and

blocking this reflex with muscarinic antagonists improves lung function in adults[182,183] and children[184,185] with severe asthma.

C2.2 Neural Mechanisms of Airway Hyperreactivity

Heightened reflex bronchoconstriction and airway hyperreactivity suggests airway nerves are dysfunctional in patients with asthma. Inflammation sensitizes nerves, increases expression and release of neurotransmitters, and impairs degradation and inhibitory feedback loops that limit further activity (Figure 2).

C2.2.1 Secreted Mediators

Afferent nerves are required for airway hyperreactivity[186] and allergen sensitization and exposure increase sensory nerve sensitivity[187,188]. Airway inflammation increases expression of many mediators that activate sensory nerves, such as histamine[176], serotonin[179], adenosine triphosphate[189,190], leukotrienes[191], prostaglandins[192], bradykinin[193], neurotrophic factors (see neurotrophic section below), protons[194,195], protease receptor agonists[196-198], interleukins[199,200], and TNFα[201]. Eosinophil secretion of cationic protein, major basic protein, and eosinophil peroxidase also activate pulmonary sensory C-fibers[202]. These mediators can directly cause nerve depolarization, but many also sensitize nerves to increase responses to subsequent stimuli[191,196,201,203-205].

C2.2.2 Nerve Structure

Heightened airway innervation promotes airway reactivity to sensory afferent stimulation[6], suggesting structural alterations to nerves increase reflex bronchoconstriction. Eosinophils increase airway innervation and reactivity *in vivo*[206] and promote neurite outgrowth and branching of cultured dorsal root ganglia[207]. Airway innervation is also increased in biopsies from humans with eosinophilic asthma compared to individuals without asthma, demonstrating eosinophils have similar effects on nerve structure in humans[208]. Eosinophil-nerve interactions are not isolated to lungs as patients with atopic dermatitis have significant dermal eosinophilia with associated increases in sensory innervation[207]. Mouse models of atopic dermatitis also have increased sensory innervation and eosinophil-dependent itch[209].

C2.2.3 Substance P

Airway inflammation increases expression of substance P, a sensory neurotransmitter that contributes to asthma pathology.

Innervation targets

Substance P is an 11 amino acid peptide[210] encoded by the preprotachykinin gene, which also encodes two other tachykinins, neurokinin A and neurokinin B[211-214], and each bind to their corresponding receptors neurokinin-1 (NK1), neurokinin-2 (NK2), and neurokinin-3[215,216]. Substance P also binds Mas-related g-coupled protein receptors B and A1 in addition to NK1[217,218]. Nerves that

express substance P innervate airway epithelium, submucosal glands, smooth muscle, vasculature, and parasympathetic ganglia[219-223]. Most airway epithelial nerves express substance P[220] with cell bodies residing in jugular ganglia[152,224]. Few nerves located in airway smooth muscle express substance P[220] with cell bodies of these fibers likely originating from intrinsic airway ganglia[219,225-227]. Dorsal root ganglia also express substance P and supply intrapulmonary innervation[222,223,228].

Regulation of expression, release, and degradation

Nerve growth factor (NGF) increases substance P expression[229] and controls development of substance P expressing sensory nerves[230]. NGF is likely derived from innervation targets as switching nerve receptor fields can increase or decrease substance P expression[231,232]. The inflammatory cytokines IL-1β[233] and TNFα[234,235] (via induction of IL-1β and leukemia inhibitory factor[235,236]) also increase substance P expression. Substance P release from neurons occurs in response to many stimuli, including potassium[237], capsaicin[238], bradykinin[239], serotonin[240], histamine[241], protease-activated receptor[242], acid-sensing ion channel 1[194], and transient receptor potential channel A1 channel[243] activation.

Neutral endopeptidase and angiotensin-converting enzyme[244] degrade substance P and inhibition of either enzyme potentiates airway responses to substance P[245-248]. Airway epithelium[249,250], vascular endothelium[251], and immune cells[249,252] express neutral endopeptidase. Suppression of neutral endopeptidase

activity contributes to airway hyperreactivity and occurs after viral infection[253], allergen[254,255] and oxidant exposure[256,257]. However, neutral endopeptidase also degrades other neurotransmitters, such as neurokinin A[258] and bradykinin[259], thus potentiation of airway responses after inhibition of neutral endopeptidase cannot solely be attributed to substance P.

Airway effects

Stimulation of airway sensory nerves causes local reflex release of substance P[260] that increases mucus secretion[261-263] and vascular leakage[260,264,265], contributing to airway obstruction and edema. Plasma leakage induced by substance P involves both mast cell-dependent and -independent mechanisms[266,267]. Substance P causes mast cell degranulation[268-270] and mast cell-dependent leakage depends on histamine[266]. Substance P also recruits[271-273] and activates other immune cells. Substance P is not restricted to nerves as eosinophils[274], dendritic cells[275]and macrophages[276] express substance P. Whether endogenous or nerve-derived, substance P causes immune cell secretion of inflammatory cytokines[276-278], oxidized lipids[279], granule proteins[280], and augmentation of effector functions[281].

Substance P causes bronchoconstriction, both directly by binding NK1 receptors on airway smooth muscle[282], and indirectly, by potentiating nerve function. Substance P contracts smooth muscle[283-286] and increases airway resistance[287], however it is less potent than other tachykinins[285,287] in directly causing

bronchoconstriction. Substance P expressing nerves innervate airway parasympathetic ganglia and increase cholinergic bronchoconstriction[288] by potentiating synaptic transmission through ganglia[289,290], depolarizing neurons[291], and facilitating acetylcholine release[292-294].

Substance P and Asthma

Individuals with asthma have elevated substance P in airway lavage fluid[295,296], airway nerves[297], and peripheral blood[298] compared to individuals without asthma. Allergen exposure increases airway nerve substance P expression[299-303] and airway sensitivity to substance P[254]. Exogenous NGF[304,305], in the absence of allergen exposure, mimics allergen-induced increases in nerve substance P and inhibition of nerve growth factor[299] or its receptor TrkA[300] can prevent allergen-induced substance P expression. Blocking substance P receptors prevents allergen-induced airway hyperreactivity[306] in animal models of asthma, however a dual NK1/NK2 antagonist paradoxically worsened allergen-induced airway responses in patients with asthma[307]. Other peptides bind to NK1 receptors[308] and Mas-related g-couple protein receptors cause airway hyperreactivity[309], thus it is presently unclear whether NK1 antagonists failed because the role of substance P in human asthma differs from animal models or if substance P-mediated airway hyperreactivity involves multiple pathways.

C2.2.4 Calcitonin-gene related peptide

Substance P positive airway nerves co-express and co-release calcitonin-gene related peptide (CGRP)[310,311], a neuropeptide with opposite effects on airway physiology and immune cell function compared with substance P. Two isoforms of CGRP exist, α and β. Tissue-specific alternative RNA splicing of the calcitonin gene[312,313] regulates CGRP-α expression, while CGRP-β[314] is encoded by a separate gene, but differs from the α form by only one amino acid. Most laboratory techniques cannot distinguish between CGRP-α vs. -β, thus subsequent sections will generically refer to CGRP with the notable assumption that both isoforms behave similarly.

Innervation Targets, Expression, Release, and Degradation

Substance P and CGRP expressing nerves innervate similar targets and share remarkably similar pathways regulating release and degradation. Airway C-fiber nerves from both vagal ganglia[311,315] and dorsal root ganglia[315,316] express CGRP, providing innervation of vasculature[313,315], epithelium[315,317], and parasympathetic ganglia[318]. Intrinsic airway ganglia and neuroepithelial bodies (chemosensors in contact with the airway lumen) also express CGRP[311,317,319]. Secretion of CGRP occurs in response to potassium[320], capsaicin[321], histamine[322], bradykinin[322], prostaglandins[323], or transient receptor potential channel A1[324,325] activation and is inhibited by serotonin[326,327]. CGRP binds a G protein-coupled receptor, and in complex with an accessory protein required for ligand specificity[328], stimulates cyclic adenosine monophosphate production[329-331]

and cAMP response element binding (CREB) phosphorylation[332]. Receptor desensitization or degradation by neutral endopeptidase[321,333] and mast cell tryptase[334,335] terminates CGRP signaling. TNFα[336], IL-1β[337,338] and the neurotrophic factors NGF[229,339] and glial-cell line derived neurotrophic factor (GDNF)[340] increase CGRP expression.

Airway Effects

Allergen sensitization and exposure increases airway lavage CGRP[341], however studies are mixed whether this reflects a depletion of stored CGRP[342] or an increase in CGRP synthesis[343,344]. Stimulation of airway sensory nerves causes local reflex release of CGRP, resulting in profound vasodilation[345,346], suppression of immune cell responses, and inhibition of bronchoconstriction. CGRP does not cause vascular leakage, but can potentiate substance P-mediated edema[347-349], presumably due to increased local blood volume and competition for degradation by neutral endopeptidase. However, substance P limits CGRP-induced vasodilation[350] by inducing mast cell degranulation and subsequent degradation of CGRP by tryptase.

CGRP suppresses inflammation and allergen sensitization. CGRP inhibits macrophage[351] and dendritic cell antigen presentation[352-354], thus reducing allergen-induced T cell expansion. CGRP also promotes IL-10 secretion and development of tolerogenic T regulatory cells[352] to further suppress allergen-induced inflammatory responses. In addition to airway nerves, alveolar type II

cells secrete CGRP[355]. Disruption of epithelial barriers after allergen exposure increases dendritic cell access to antigens and epithelial cytokines promote dendritic cell trafficking to lymph nodes. CGRP protects against epithelial injury[356] and promotes epithelial cell proliferation[357], migration[358] and healing[359], and thus may suppress allergen sensitization indirectly by maintaining epithelial barrier integrity.

CGRP does not cause bronchoconstriction[311,321,360] and actually blocks serotonin[361] or substance P-induced contraction[362]. Inhaled CGRP suppresses allergen-induced airway hyperreactivity[342], potentially by hyperpolarizing airway ganglia[318] and inhibiting acetylcholine release[363,364]. Paradoxically, genetic deletion of CGRP in mice also prevents allergen-induced airway hyperreactivity[365]. However, CGRP knockout mice have higher sympathetic activity[366] at baseline, which could suppress airway hyperreactivity through activation of β_2-adrenergic receptors. Inducible CGRP knockout mice will be needed to further clarify the role of CGRP in airway hyperreactivity.

C2.2.5 M2 Muscarinic Receptors

Parasympathetic nerves control airway tone by releasing acetylcholine, which binds to M_3-muscarinic receptors and causes smooth muscle contraction[158]. Released acetylcholine also binds inhibitory M_2-muscarinic receptors located on postganglionic nerves[159,160] to limit further acetylcholine release. M_2 receptors are

inhibited in individuals with asthma[367,368] and after allergen sensitization and exposure[42,369-372] in animal models, thus increasing nerve-mediated bronchoconstriction.

Eosinophils inhibit M_2-muscarinic receptor function. Eosinophils cluster around airway nerves in patients with asthma[373] and after allergen exposure[42,373]. Airway nerves actively recruit eosinophils by releasing eotaxin-1[372], a potent inducer of eosinophil migration, and bind eosinophils through complementary integrin-cell adhesion molecule interactions[374]. Eosinophils are enriched in major basic protein, an allosteric antagonist of parasympathetic nerve M_2 receptors[375] and degranulate after binding airway nerves[374]. Decreasing airway eosinophils, eosinophil recruitment[370,372], adhesion[371], or blocking major basic protein[42] protects M_2-muscarinic receptor function and prevents allergen-induced airway hyperreactivity.

C3. Neurotrophic factors

Neurotrophic factors are a large family of growth factors required for nerve development, survival, and response to injury or inflammation. Two main groups of neurotrophic factors exist, including the classic neurotrophin family comprised of NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4, and a second group comprised of GDNF and related members neurturin, artemin and persephin. The following section highlights the role of NGF, BDNF, and GDNF in asthma pathogenesis, focusing on how they affect the

development of lung innervation, postnatal nerve function, and immune system homeostasis.

C3.1 Nerve growth factor

NGF is required for sympathetic and nociceptive sensory nerve development[376,377] and is secreted by innervation targets during embryonic development to promote neurite growth[378-380] and increase innervation density[381,382]. After development, NGF maintains sensory and sympathetic nerve phenotypes by supporting neurotransmitter expression[230,383]. NGF is synthesized as a precursor[384] and proteolytically processed in both intracellular[385] and extracellular compartments[386]. Upon secretion, NGF binds two receptors[387] with different affinities. The high-affinity TrkA receptor[388,389] has tyrosine kinase activity[390] and binding of NGF activates Ras/mitogen-activated protein kinase[391-393], Akt, or phospholipase C signaling cascades[394]. The low-affinity $p75^{NTR}$[395,396] binds all neurotrophins and can activate NF-κB signaling[397] as well as other signaling pathways depending on interactions between $p75^{NTR}$ and co-receptors[398]. NGF's effect on neurons depends on the balance between TrkA and $p75^{NTR}$ expression and intracellular signaling[399-401], the stage of nerve development[402,403], and conversion of proNGF into mature NGF[386].

NGF is secreted by numerous cells in the lungs and affects non-neuronal tissues in addition to its requirement for nerve development. The airway epithelium is the richest source of NGF[404,405] and expression increases in response to viral

infection[406], allergen exposure[299], or the inflammatory cytokines IL-1β or TNFα[407-410]. In addition to airway epithelium, infiltrating immune cells, fibroblasts, smooth muscle, and blood vessels all secrete NGF[404,405,411,412]. NGF is increased in serum and lung lavage of individuals with asthma compared to those without asthma, and levels increase further after allergen exposure[404,405,413-415]. NGF contributes to all defining features of asthma, including airway hyperreactivity, inflammation, and structural remodeling.

NGF has both acute and chronic effects on airway hyperreactivity. Elevated NGF in the absence of inflammation increases epithelial innervation and induces airway hyperreactivity[6]. NGF causes airway hyperreactivity by promoting sensory innervation during development and by sensitizing neurons to stimuli acutely. NGF increases nerve excitation and airway contraction[6,416-419] likely by increasing sensory nerve expression of both receptors[420-422] and neurotransmitters[229,304,305] that contribute to airway hyperreactivity. NGF also augments synaptic transmission in parasympathetic nerves[423], which ultimately control airway smooth muscle contraction through release of acetylcholine. Thus, NGF promotes airway hyperreactivity by affecting both afferent and efferent limbs of airway innervation.

Targeting NGF or its receptors reduces airway hyperreactivity by suppressing nerve excitation (detailed above), airway inflammation[424-429], and remodeling. Mast cells[430], T lymphocytes[431,432], and eosinophils both secrete and respond to

NGF. Mast cells release NGF after IgE receptor activation[433] and respond to NGF by releasing serotonin[434,435] and histamine[436] to activate airway nerves and smooth muscle. Eosinophils both secrete NGF[437] and augment epithelial NGF secretion[438]. After NGF exposure, eosinophil hematopoiesis increases[439], cells survive longer[438,440] and degranulate to release eosinophil peroxidase[441]. Airway remodeling occurs in response to airway inflammation, but NGF directly promotes subepithelial collagen deposition[442] and smooth muscle proliferation[443], causing increased baseline airway resistance and reduced lung compliance[442].

C3.2 Brain-derived neurotrophic factor

BDNF is highly expressed in the central nervous system where it regulates the development and function of several nerve populations. BDNF's effects on the central nervous system will not be reviewed here. It is important to note, however, that *in vitro* studies evaluating how BDNF affects nerve function predominantly use nerves isolated from the central nervous system. Similarly, *in vivo* studies on the peripheral actions of BDNF occur in the context of significant central nervous system dysfunction and it is difficult to dissociate the pathology between the two systems.

While expressed at much lower levels in the periphery[444], BDNF is required for mechanoreceptor sensory nerve development of the viscera and skin[445-451] and is secreted by innervation targets to promote neurite growth[452]. After development BDNF increases survival of additional nerve populations, namely motor neurons,

during injury or inflammation[453-456]. BDNF is synthesized as a precursor and released in response to nerve activity[457] and inflammation[458]. proBDNF is converted to mature BDNF by extracellular proteases[457] and subsequently binds TrkB[459-461] and p75^{NTR}[462]. The high-affinity TrkB receptor has tyrosine kinase activity and binding of BDNF activates Ras/mitogen-activated protein kinase, Akt, or phospholipase C signaling cascades[394]. The low-affinity p75^{NTR} binds all neurotrophins and can activate NF-κB as well as other pathways depending on interactions with co-receptors. BDNF's effect on neurons depends on the balance between TrkB and p75^{NTR} expression and signaling[463], expression of different TrkB isoforms[464], stage of nerve development[465], and conversion of proBDNF into mature BDNF[457,463].

BDNF is secreted by numerous cells in the lungs and affects non-neuronal tissues in addition to its requirement for nerve development. Respiratory epithelium[466], smooth muscle[467], and infiltrating immune cells[466,468] are the primary sources of BDNF and secretion of BDNF increases after exposure to allergen[414,469] or the inflammatory cytokine TNFα[467,470]. BDNF is increased in serum and lung lavage of individuals with asthma compared to those without[415] and levels correlate with asthma severity in both adults[471] and children[472].

Unlike NGF, far less is known about how BDNF increases disease severity and contributes to specific pathogenic features of asthma. Most data show that airway smooth muscle both secretes and responds to BDNF. BDNF stimulates

smooth muscle proliferation[473] and augments contractility[474,475]. Nerve activity and intracellular calcium increase BDNF expression in neurons[476-478]. It is plausible similar mechanisms exist in airway smooth muscle to account for enhanced BDNF expression in individuals with asthma[467]. However, TrkB desensitization and down-regulation occurs with chronic BDNF exposure[479], so it is unclear how long-term elevations in BDNF contribute to smooth muscle pathology.

There are sparse data on how BDNF affects airway hyperreactivity and inflammation. BDNF increases expression of nerve ion channels[469,480-482], axon arborization[483,484], and synapse formation[484] to promote excitation, potentiates parasympathetic nerve synaptic action potentials[485], and increases neurotransmitter release[486]. However, the consequences of these effects with regard to airway hyperreactivity are unknown. Similarly, how BDNF contributes to airway inflammation is also unknown. Eosinophils secrete[437] and respond to BDNF with release of eosinophil peroxidase[487], but the functional significance of this interaction and whether BDNF affects other immune cells is unknown.

C3.3 Glial cell line-derived neurotrophic factor

GDNF is required for innervation of the gut[488,489] where it promotes nerve precursor proliferation and differentiation[490,491]. After development GDNF supports parasympathetic, sympathetic, motor and proprioceptive sensory

nerves[492-495]. GDNF binds the GFRα-1 receptor[496-498] and initiates RET tyrosine kinase signaling[499-501].

The role of GDNF in asthma pathogenesis has not been studied. All sensory neurons in the airways express the GFRα-1 receptor[502] and GDNF can promote parasympathetic lung innervation[503]. Similar to BDNF, GDNF can increase sensory nerve expression of neurotransmitters and receptors[504-506] to promote excitation and enhance neurotransmitter release[507]. However, the functional consequences of these changes are unknown. GDNF is also expressed in lung homogenates[508], but it is unclear what cell types express it or how expression and secretion is regulated in the airways.

D. Airway Nerve Development

Lung function trajectories are established during childhood and strongly predict adult lung function and respiratory morbidity. Lungs are not fully mature at birth and development continues until young adulthood, thus postnatal environments strongly influence lung development and attainment of optimal lung function.

D1. Prenatal Airway Nerve Development

Lung development begins at day 9 of mouse gestation and day 28 of human gestation, comprising five phases that are defined by morphological appearance of lung parenchyma. Embryonic (mouse: E9.0-E12.5) lung development begins when anterior foregut endoderm evaginates to form the trachea and lung buds,

and proceeds with tracheal separation from the esophagus and lung bud enlargement. Initiation of a stereotyped branching program[509] to generate complex tree-like conducting airways defines pseudoglandular (E12.5-E16.5) lung development. Airway branching ceases during cananicular (E16.5-E17.5) lung development and terminal ends narrow to form terminal bronchioles. Saccular (E17.5-P4) lung development involves formation of small sacs at the end of terminal branches, along with respiratory bronchiole and alveolar duct formation. Alveolarization (P4-P21) involves successive divisions of sacs into smaller subunits to give rise to many thousands of alveoli and functional gas-exchange units.

Parasympathetic ganglia appear during pseudoglandular development as flat patches of neural precursors that originate from neural crest cells[510,511] and travel along extending vagal nerves[512,513]. Ganglia form around nerve and airway bifurcations and condense, enlarge, and become more spherical in appearance throughout gestation[512,514-516]. Parasympathetic ganglia express detectable choline acetyltransferase[516] and airways contract in response to electrical stimulation[517,518] during cananicular development, suggesting nerves are also functional at this stage. Vagal afferent innervation, originating from both neural crest cells (jugular ganglia) and epibranchial placodes (nodose ganglia)[519], develops in parallel with calcitonin-gene related peptide and substance P expressing nerves detected during pseudoglandular development[520,521].

Molecular control of lung nerve development is poorly understood. What guides neural precursor migration, proliferation, and subsequent parasympathetic ganglia network formation in the lung is unclear. GDNF can guide neuron migration *in vitro*[503], however mice deficient in GDNF exhibit no defects in lung innervation[511]. This sharply contrasts with neural crest cell development in the gut, since gastrointestinal innervation fails to develop in mice deficient in GDNF, suggesting organ specific molecular regulation of vagal innervation. Similarly, netrin and deleted in colorectal cancer (Netrin/DCC) signaling guides neural crest cells migration into the gut[522], but deletion of DCC has no effect on lung nerve development[523]. Target-derived NGF likely regulates sensory innervation and expression of substance P and CGRP, but airway-specific deletion of NGF and subsequent effects on innervation have not been studied.

D2. Postnatal Airway Nerve Development

Airway innervation continues to develop postnatally, with increases in sensory innervation[524-528] and sensitivity to contractile agonists[529-531], and decreases in relaxant innervation[530,532]. Innervation comparable to adults is reached within a few weeks after birth for mice. Environmental insults during this critical period permanently alter airway innervation and promote airway hyperreactivity later in life. Postnatal house dust mite or ozone exposure in infant rhesus monkeys increases epithelial innervation at one year of age[533] and is associated with airway hyperreactivity at three years of age[534]. Similarly, postnatal ovalbumin[535] or cigarette smoke[536] exposure increases airway innervation with subsequent

airway hyperreactivity in mice. Increased innervation after allergen or cigarette smoke depends on neurotrophic factors that reach a peak in expression during postnatal alveolarization[535,537]. The postnatal period represents a particularly sensitive time period, since environmental exposures later in life do not alter lung innervation and cause persistent airway hyperreactivity as they do in neonates[526,529,536].

Neonatal airway immune responses to environmental insults are intrinsically skewed towards type 2 inflammation. The first breath of a neonate induces IL-33 secretion from airway epithelial cells with high constitutive secretion of IL-33 throughout the postnatal period[538]. IL-33 secretion activates a cascade of airway type 2 innate lymphoid cell expansion, IL-5 and IL-13 secretion, and recruitment of peripheral eosinophils into the airway[538-540]. This postnatal influx of eosinophils and type 2 innate lymphoid cells occurs naturally and cells decline to adult levels after a couple weeks in mice, however it creates a vulnerable window for environmental insults to impact airway immunity and nerve function. Allergen exposure increases cytokine production, type 2 lymphocyte polarization and expansion, eosinophilia and IgE production more in neonates than in adults[539,540]. The above studies were conducted in mice and whether a similar influx of type 2 innate immune cells into human lungs occurs is unknown. Hospitalized premature infants consistently develop postnatal peripheral eosinophilia[541-544], which is presumed to be pathologic, but could actually reflect normal maturation and recruitment. The National Heart, Lung and Blood Institute recently funded a

consortium (LungMAP)[545] that is tasked with creating a molecular atlas of the developing human lung, with a particular focus on postnatal alveolarization, and will hopefully serve as a resource to answer such questions.

E. Summary

Reflex bronchoconstriction involves coordinated activation of afferent and efferent nerves (Figure 1). Blocking this reflex with muscarinic antagonists improves lung function in severe asthmatics, demonstrating the importance of nerve function in asthma pathogenesis. Several mechanisms of nerve-mediated airway hyperreactivity are known (Figure 2). Airway inflammation increases cytokines, neurotrophic factors, and eosinophil-derived proteins that either cause nerve depolarization directly or increase sensitivity to subsequent depolarizing stimuli. Allergen exposure also increases expression of the neurotransmitter substance P, which augments inflammatory responses and synaptic transmission through efferent, parasympathetic ganglia. Finally, recruited eosinophils inhibit M2 muscarinic receptors, thus potentiating acetylcholine release from efferent nerves. All of these changes together cause nerve-mediated airway hyperreactivity.

Maternal asthma increases the risk of childhood asthma. Children born to parents with asthma have airway hyperreactivity at birth, suggesting *in utero* development of airway responses. However, it is unknown whether maternal asthma affects fetal airway nerve development.

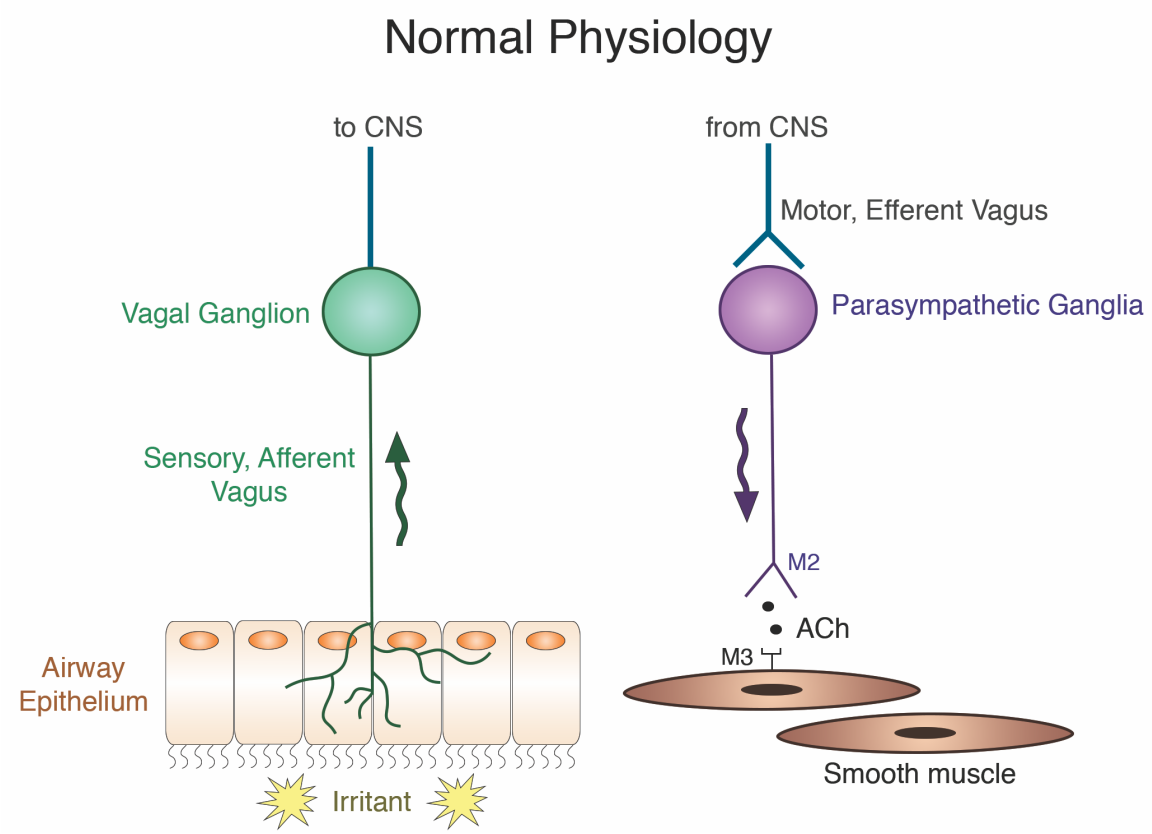

Figure 1. Schematic of reflex bronchoconstriction. Inhaled irritants activate afferent epithelial sensory nerves with cell bodies located in the nodose-jugular ganglia of the vagus. Efferent nerves contained within the vagal nerve synapse on parasympathetic ganglia embedded within the lungs, releasing acetylcholine (ACh) from postganglionic parasympathetic nerves which contracts airway smooth muscle via M_3-muscarinic receptors (M3). M_2-muscarinic receptors (M2) located on pre-synaptic postganglionic nerves inhibit further acetylcholine release.

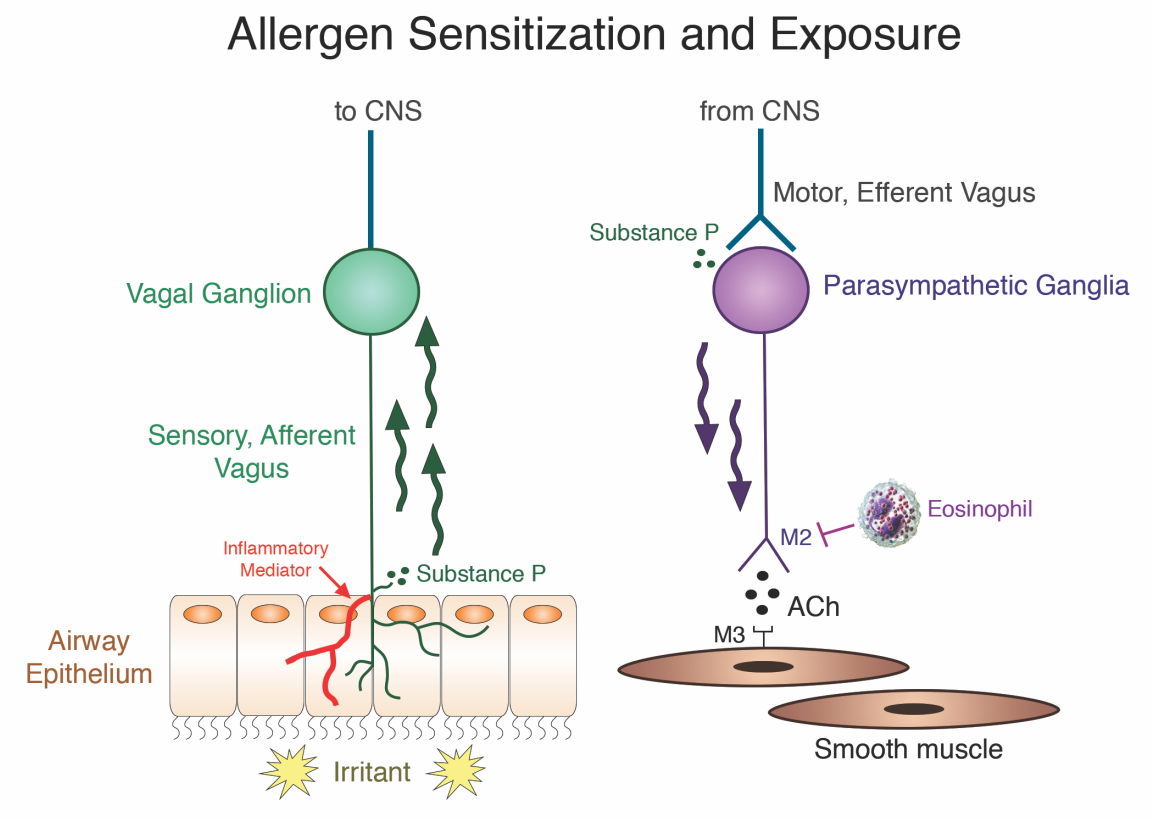

Figure 2. Neural mechanisms of airway hyperreactivity. Airway inflammation potentiates both afferent and efferent nerve activity to cause airway hyperreactivity. Secreted mediators, such as inflammatory cytokines, neurotrophic factors, and eosinophil-derived proteins, sensitize afferent sensory nerves (red) and potentiate responses to inhaled stimuli. Allergen exposure increases nerve substance P, which subsequently augments synaptic transmission through efferent, parasympathetic ganglia. Not pictured above is allergen-induced inhibition of neutral endopeptidase activity, which further increases airway substance P. Recruited eosinophils secrete major basic protein, which antagonizes M_2-muscarinic receptors (M2), thus disrupting inhibitory feedback loops and potentiating acetylcholine release.

F. Hypothesis

I hypothesize that maternal allergen exposure causes structural and functional changes in airway nerves of offspring that are mediated by eosinophils and lead to airway hyperreactivity.

CHAPTER 2. General Methods

A. Model Rationale

A1. House dust mite

The house dust mite (HDM), dermatophagiodes pteronyssinus, is allergenic[546] and the majority of individuals with asthma are sensitized to it[547]. Exposure to house dust mite elicits robust eosinophilia, airway hyperreactivity, and type-2 cytokine production in animal models of asthma[548]. HDM is also more clinically relevant than ovalbumin, thus, I chose HDM sensitization and exposure as a model for type-2 high, eosinophilic atopic asthma.

A2. Serotonin-induced reflex bronchoconstriction

Methacholine[173,174], histamine[175,176], bradykinin[177], capsaicin[178], and serotonin[179] all evoke reflex bronchoconstriction. Methacholine and high-dose histamine also directly contract airway smooth muscle. Methacholine and serotonin reproducibility cause reflex bronchoconstriction in a mouse, while results with other contractile agents are variable[549] or contain logistical challenges (ex: capsaicin evokes cough in laboratory personnel). Thus, I chose serotonin to study reflex bronchoconstriction in mice.

A3. Mice

I used C57Bl/6 mice for all studies due to the ease of genetic manipulation (ex: eosinophil knock-out), availability of reagents, equipment, and techniques (ex: respiratory physiology), cost and resource burden, and ability to conduct

transgenerational experiments in a reasonable timeframe. C57Bl/6 mice have a less “allergic” phenotype than BALB/c mice, with less IgE, IL-5, and IL-13 production after allergen exposure and reduced correlation between eosinophils and airway hyperreactivity[550,551], but were required since eosinophil deficient [(-)Eos] and IL-5 transgenic mice (IL5tg) are on a C57Bl/6 background.

Hemizygous IL5tg mice express IL-5 under a CC10 promoter (this strain is more commonly referred to as NJ1726) (11). Three models of elevated IL-5 exist. IL5tg mice were chosen due to selective lung IL-5 expression, as opposed to dual expression of IL-5 and eotaxin-2[552]. Mice with overexpression of IL-5 driven by a T cell promoter[553] have elevated peripheral IL-5, and would have been suitable for fetal development studies (Chapter 3), but not for airway inflammation phenotype studies (Chapter 5).

Eosinophil-deficient mice [(-)Eos] were generated by expressing diphtheria toxin under the eosinophil peroxidase promoter (12). Five different eosinophil deficient mice exist. (-)Eos mice (more widely known as PHIL mice) were chosen because this is the best-characterized strain with selective and complete ablation of eosinophils. Genetic deletion of both major basic protein and eosinophil peroxidase leads to a selective and dramatic reduction in eosinophils, but not complete ablation[554]. Genetic deletion of IL-5 or the IL-5 receptor does not completely ablate eosinophils and also impairs B cell development[555]. GATA-1

knockout mice[556] are also deficient in eosinophils, but have defective basophil development[557].

A4. Comparison with human asthma

No animal model perfectly recapitulates human asthma, since few animals naturally develop asthma (the exception being some horse and dog breeds). Therefore, asthma models are best suited for studying specific symptoms or components of disease, thus I focused on airway eosinophils and airway hyperreacitivity. A few notable differences exist between mouse and human airway physiology. Mouse lungs have fewer respiratory bronchioles and airway generations, larger airway caliber, greater chest wall compliance, and a paucity of submucosal glands compared with humans[558]. Mice also respond to fewer contractile agents than humans. Bronchoconstriction in humans occurs after exposure to histamine[559,560], methacholine[560], capsaicin[178], bradykinin[561], leukotrienes[562], and serotonin (assessed with an antagonist, not directly)[563]. Serotonin and methacholine produce consistent bronchoconstriction in mice, while histamine, substance P, prostaglandins, and leukotrienes produce variable responses[549].

Mice are also born at an earlier stage of lung development than humans, completing both the saccular stage and alveolarization in postnatal environments compared with just alveolarization in humans. Airway nerve development is poorly understood, thus it's unclear whether postnatal exposures at an earlier

stage of lung development in mice impacts nerve function differently than the same exposure in humans. What is clear is that early life insults in both mice[535,536] and humans[564] leads to airway hyperreactivity later in life, but novel, noninvasive methodologies are needed to test if nerve changes observed in mice also occur in humans.

A comprehensive review on the similarities and differences between mice and human eosinophils has been published and covers cell surface markers, protein expression, morphology, recruitment and activation signals, effector functions, and regulation of survival vs. death[565]. Mouse and human eosinophils share many similarities, but an important difference pertains to eosinophil-nerve interactions in asthma. Airway biopsies and lavage fluid from humans with asthma contain abundant major basic protein[566,567], evidence of extensive eosinophil degranulation. Mouse eosinophils do not degranulate as easily or in response to the same stimuli as human eosinophils. Degranulation is not necessary to drive lung pathology in mice[41] and little major basic protein is seen in lungs from allergen exposed mice[568]. Major basic protein is an allosteric antagonist of M_2-muscarinic receptors[375] and blocking major basic protein prevents airway hyperreactivity in guinea pig models of asthma[42]. However, major basic protein knockout does not prevent airway hyperreactivity in mice after allergen exposure[43]. Thus, mouse models do not perfectly recapitulate the role of eosinophils in promoting airway hyperreactivity in humans.

B. Transgenic Mice and Genotyping

The Oregon Health & Science University's IACUC committee approved all animal experiments. Mice were housed and bred at OHSU with ad libitum access to food and water on a 12-hour light and dark cycle. All mice were on a C57Bl/6J background and both male and female mice were used for experiments per National Institute of Health guidelines. Wild-type (WT) mice were purchased from Jackson Laboratories and transgenic mice were a gift from Dr. James Lee (Mayo Clinic, Scottsdale, Arizona).

Mice were genotyped by PCR and gel electrophoresis. Briefly, ear tissue was collected from adult mice under 5% isoflurane anesthesia or the distal tail was removed from fetuses. HotSHOT DNA extraction was performed with alkaline lysis reagent (125 µL of 10N NaOH, 20 µL of 0.5M EDTA) followed by neutralization buffer (325 mg Tris-HCl). After DNA extraction, EconoTaq PCR master mix plus green (Lucigen Cat. 30033) was added to 1 µM (-)Eos and 0.1 µM 18s or 0.2 µM IL5tg and 0.2 µM 18s primers (see below) and run through a PCR reaction using a 96 well Veriti Thermal Cycler (Applied Biosystems). After a 5 minute 94°C hot start, PCR denaturation started at 94°C for 30 seconds, followed by annealing step at 53°C for (-)Eos or 56.5°C for IL5tg for 1 minute duration, and extension at 72°C for 1 minute. Both PCR reactions were cycled 30 times, then ran on a 2% agarose gel.

(-)Eos Primers:

Forward - 5'-AAG TAT GAT GGG GGT GTT TC-3'

Reverse - 5'-GAG CGG GTT TTC ATT ATC TAC-3'

IL5tg Primers:

Forward - 5'-CAG TGC TTG ACT TTA AAG AGG-3'

Reverse - 5'-TGG CAG TGG CCC AGA CAC AGC-3'

18s Control Primers:

Forward - 5'-GTA ACC CGT TGA ACC CCA TT-3'

Reverse - 5'-CCA TCC AAT CGG TAG TAG CG-3'

C. Allergen sensitization and exposure

For maternal allergen exposure studies (Chapter 3), nulliparous female C57Bl/6J mice were sedated with 5% isoflurane and intranasally exposed to 25 µg HDM (dissolved in 25 µL PBS; LPS content 83250 EU/vial; Greer Laboratories) or vehicle (PBS) once daily, 5 days per week, for 4 weeks. At the end of the 4th week of challenge, female mice were mated with male mice. On the 5th week until delivery, pregnant female mice were manually restrained and intranasally exposed to 25 µg HDM (dissolved in 25 µL PBS) or vehicle (PBS). In pilot experiments, daily isoflurane sedation caused fetal demise, thus manual restraint without sedation was required for daily HDM or vehicle exposures.

For offspring or adult mice allergen exposure (Chapter 3 and 5), mice >8 weeks of age were anesthetized with 5% isoflurane and sensitized intranasally with vehicle (PBS) or 50 µg of HDM (dissolved in 25 µl PBS) on protocol days 0 and 1, followed by challenge with vehicle or 25 µg HDM (dissolved in 25 µL PBS) on days 14-17. Animals were sacrificed on day 18 for analysis. Inhaled anesthetics (desflurane, isoflurane) activate sensory nerve ion channels[569,570] and can affect tachykinin physiology[571], thus offspring studies include none sedated, none exposed controls. For chronic allergen exposure (Chapter 5), mice were anesthetized with 5% isoflurane and challenged with vehicle or 25 µg HDM (dissolved in 25 µL PBS) five days per week for 8 weeks. Animals were sacrificed on day 57 for analysis.

D. Airway physiology

Mice were sedated with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.), tracheotomized, and mechanically ventilated via a 21-gauge catheter. The ventilator system consisted of a pneumotachograph to measure airflow (ML141, AD Instruments), a pressure transducer, metering valves (inspiratory time 175 ms, expiratory time 300 ms), two expiratory water columns for positive end-expiratory pressure (2 cm H20) and deep-inhalation (25 cm H20), in-line nebulizer (AeroNeb) and LabChart Pro acquisition software. Mice were ventilated with 100% oxygen at 125 breaths/min with a 0.2 mL tidal volume and paralyzed with succinylcholine (10 mg/kg i.p.) to eliminate respiratory effort. Core body temperature was maintained at 36.9°C with a homeothermic blanket. A 3-lead

electrocardiogram monitored heart rate and rhythm and a pulse oximeter recorded oxygen saturation.

Airway resistance was calculated as the difference between peak inspiratory pressure and plateau pressure during an end inspiratory pause, divided by airflow (Resistance = P_{peak}-$P_{plateau}$ / flow). Baseline airway resistance was measured in response to 10 µl nebulized PBS vehicle followed by escalating concentrations of serotonin (10 - 1,000 mM). Resistance after each dose of serotonin was expressed as fold change over resistance to aerosolized PBS.

E. Drugs

Drug information is summarized in table 1 and includes indication, dose and route of administration, dissociation and inhibitory constants when known, source, and relevant references.

E1. Muscarinic Agonist and Antagonists

Some mice were treated with the muscarinic antagonist atropine (1 mg/kg i.v. in PBS), before inhaled serotonin to test if bronchoconstriction involved activation of M_3 muscarinic receptors by acetylcholine. To test if M_2-muscarinic receptors were functional, gallamine (an M_2-muscarinic receptor antagonist; 1 mg/kg i.v. dissolved in PBS) was administered to some mice before inhaled serotonin. For methacholine experiments, both vagus nerves were isolated and cut bilaterally

before mice were exposed to escalating concentrations of inhaled methacholine (1 - 100 mM).

E2. Neurokinin Receptors Agonists and Antagonists

For NK1 experiments, the competitive NK1 antagonist CP99994 (1 µg i.p., 20 µg i.p., 60 µg i.v., 20 µg inhaled., or 60 µg inhaled; dissolved in PBS) was given 15 minutes before measuring airway responses to serotonin. To test if NK1 activation caused bronchoconstriction directly, mice were administered phosphoramidon (a neutral endopeptidase inhibitor, 2.5mg/kg iv) and captopril (an angiotensin-converting enzyme inhibitor, 2.5mg/kg iv) and then exposed to escalating concentrations of substance P (5 - 500 µg, i.v.) or the NK1 agonist GR73632 (1 - 100 µg, i.v) and airway resistance recorded after each dose. For NK2 experiments, the NK2 antagonist GR159897 (5 µg i.v., 50 µg i.v., 5 µg i.p., 50 µg i.p., or 10, 20 or 50 µg inhaled; dissolved in DMSO or 50% EtOH) or vehicle was given 15 minutes before measuring airway responses to serotonin.

E4. Vagal Nerve Transection

For vagotomy experiments, both vagus nerves were isolated bilaterally and cut with scissors immediately before measuring airway responses to serotonin.

Table 1. Drugs used for *in vivo* studies. All drugs dissolved in sterile phosphate buffered saline (pH 7.4) unless otherwise noted.

Drug	Indication	Dose and Route	Pharmacology & References	Source
Sedatives and analgesics				
Ketamine + Xylazine	Sedation and analgesia	100 mg/kg ip 10 mg/kg ip		Par Pharmaceuticals Cos; Vet One
Pentobarbital	Euthanasia	300 mg/kg ip		Vortech
Succinylcholine	Paralysis	10mg/kg ip		Sigma
Serotonin	5-HT receptor agonist; Reflex Bronchoconstriction	10 - 1000mM; 10 µL nebulized		Sigma
Muscarinic Agonist and Antagonists				
Methacholine	Muscarinic receptor agonist	1 - 1000mM 10 µL nebulized		Sigma
Atropine	Muscarinic receptor antagonist	1 mg/kg iv		Sigma
Gallamine	M_2-Muscarinic receptor antagonist	1mg/kg iv	Ref[572,573]	Sigma
Neurokinin-1 Agonists and Antagonists				
Substance P	NK1 agonist	5-500 µg iv	Kd=0.5nM; Ref[574]	Sigma S6883
GR73632	NK1 agonist	1-100 µg iv	EC50=2nM	Tocris
CP99994	NK1 antagonist	1 µg i.p. 20 µg inhaled 60 µg inhaled 60 µg i.v.	Ki=0.25nM	Tocris
GR159897	NK2 antagonist	20 µg inhaled in 50% ethanol 20 µg inhaled in DMSO 5 µg i.p. in DMSO	Ki=0.32nM	Tocris
Evans Blue Dye	Vessel permeability	30 mg/kg iv	Ref[575]	Sigma

Phosphoramidon	NEP inhibition	2.5 mg/kg iv	Ki=2nM; Ref[575,576]	Sigma
Captopril	ACE inhibition	2.5 mg/kg iv	IC50=20nM; Ref[575]	Sigma

F. Adult Tissue Collection

Animals were assigned numbers at sacrifice to blind myself to treatment groups. If tissues were collected after airway physiology measurements, animals were still sedated and anethesetized with ketamine and xylazine, thus euthanasia was via exsanguination. If tisses were collected from nonventilated animals, mice were euthanized with pentobarbital (300 mg/kg i.p. in PBS).

Blood was collected with a heparinized 1 mL syringe and 26 gauge needle via the descending aorta. Blood was diluted 1:20 with 0.1N hydrogen chloride to lyse red blood cells and total white blood cell counts determined with a hemacytometer. Five µL of blood was smeared on a microscope slide, allowed to dry, fixed in 70%, and differential counts obtained after Wright staining.

Mice were perfused with PBS via the right ventricle. Airways were lavaged via a tracheal cannula with 500 µl PBS three times. Airway was then filled with 500 µl Zamboni's fixative, excised, and placed in 5 mL of Zamboni's fixative overnight for quantification of airway nerve architecture or histology. In some groups, the right inferior lobe was clamped to prevent infiltration of fixative, airways lavaged with Zamboni's, and the right inferior lobe removed and flash frozen. For vascular extravasation studies (Chapter 5), the airway was removed after perfusion and placed into 1 mL of formamide for 48 hours. Lavage fluid was centrifuged (2000 rpm x 10 min), and supernatants were flash frozen in liquid nitrogen, and stored at -80°C. Airway cell pellets were resuspended in PBS and total cell counts

determined with a hemacytometer. Cells were cytospun, slides fixed in 70% ethanol and differential counts obtained after Wright staining.

Spinal cord and vertebrea were transected at C7 and T12, spinal column dissected free from surrounding musculartur e, removed en bloc, and placed in Zamboni's overnight. The head was severed anterior to the ears. The remaining superior spinal column and skull were dissected free and placed in Zamboni's overnight.

G. Fetal Tissue Collection

Pregnant WT, IL5tg, HDM, and vehicle exposed mice were sedated with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) at days 18-21 of gestation, abdomen and uterus incised, and a 26-gauge needle inserted into the amniotic sac. After collecting amniotic fluid, the amniotic sac was opened, fetus removed, fetal and maternal blood collected for peripheral smears, and amniotic fluid flash-frozen in liquid nitrogen. The fetal distal tail was removed for genotyping. Blood differential counts were obtained after Wright staining.

H. Neonatal Tissue Collection

Mice were checked daily for pups and postnatal day 1 considered the first morning pups were present. Pups were euthanized on postnatal day 1 and 7 by decapitation and day 21 with pentobarbital. Airways from postnatal day 1 and day 7 pups were dissected free without prior systemic PBS perfusion and placed in

Zamboni's overnight. Postnatal day 21 pups were perfused via the right ventricle with PBS, airways excised and placed in Zamboni's overnight.

I. Lavage cytokine and neuropeptide analysis.

Lavage IL-5, IL-13, GDNF, BDNF, Substance P (all from R&D Systems), NGF (Sigma), and CGRP (Phoenix Pharmaceuticals) were measured by ELISA. Neutral endopeptidase activity was measured with a fluoremetric kit (AnaSpec).

J. Tissue Optical Clearing, Imaging, and Epithelial Nerve Modeling

Following overnight fixation, airways, dorsal root ganglia, or vagal ganglia were dissected free of surrounding tissue. Whole-mount tissues were stained as previously described[227]. Fixed tissues were washed with TBS, blocked overnight (4% normal goat [or donkey serum for choline acetyltransferase staining], 1% Triton X-100, 5% powered milk in TBS pH 7.4), immunolabeled with antibody overnight (Table 2 lists all antibodies, dilutions, species and manufacturer), washed again with TBS, and then placed in secondary antibody overnight (see Table 2). If two primary antibodies of the same species were used, tissues were washed with TBS and blocked overnight with goat anti-rabbit IgG (1:100, Jackson Immunoresearch). Tissues were then incubated in the second primary antibody overnight, washed the next day with TBS, and incubated overnight one final time in secondary. No primary and IgG controls were run in parallel. Samples were then sequentially dehydrated with 50% and 100% ethanol, placed

in benzylbenzoate:benzylalcohol (2:1) for optical clearing, and mounted in Permount-sealed chambered slides.

Images were obtained with a 780 LSM confocal microscope. Entire tracheas (complete anterior to posterior surface, vocal cords to 1500 μm inferior of the carina into the main-stem bronchi) were tile-scanned at 10x and z-stacks converted to maximum intensity projections. Total airway parasympathetic ganglia were quantified by a blinded observer. Five 63x, 1.4N.A. z-stack images extending from the posterior serosal surface through the luminal space were also taken per animal in the carina of the trachea. For assessment of nerve structure, substance P positive nerves or PGP9.5 positive nerves within the epithelial layer were manually modeled and analyzed using Filaments function of Bitplane Imaris. Each image was treated as a replicate and averaged together for one value per animal.

K. Lung sectioning and NGF staining

Following Zambonis fixation and washing with PBS, left lungs were cut in half and sent to the OHSU histopathology core for paraffin-embedding and sectioning (7 μM thick). Slides were deparaffinized, rehydrated, treated with Antigen Unmasking Solution (Vector H-3300), incubated in 3% hydrogen peroxide (1:10 in Methanol), washed with PBST (PBS with 0.05% Tween), and blocked (10% Normal Goat Serum in PBST) for one hour at room temperature. Primary antibody anti-mNGF (rabbit 1:500) was added overnight at 4°C, washed with

PBST, and placed in secondary biotinylated anti-rabbit IgG antibody (goat 1:400) for one hour at room temperature. Tissues were washed, treated with ABC reagent (Vector PK-6100 Standard) for 30 minutes at room temperature, washed with PBST, and then reacted with DAB (Vector SK-4105) for 30 seconds following immediate immersion in dH2O. Slides were dried, mounted with Cytoseal, and imaged with Zeiss ApoTome2 on AxioImager

L. Nodose-Jugular Ganglia Retrograde Tracing

Mice were anesthetized with 5% isoflurane and administered 25 µL of AlexaFluor 555 tagged wheat germ agglutinin intranasally (ThermoFischer, 10 mg/mL in PBS). Mice were euthanized 16-20 hours later with pentobarbital, vagal ganglia dissected free, and placed in Zamboni's fixative overnight. Ganglia were washed with TBS, mounted with VectaShield, and imaged with an 780 LSM confocal microscope. Fluorescently labeled neurons were counted in 3-dimensional images (Bitplane Imaris).

Table 2. Antibodies used for immunofluorescence.

Target	Species	Dilution	Source
Primary Antibodies			
Choline acetyltransferase (CHAT)	Goat	1:50	Millipore AB144P
Calcitonin-gene related peptide (CGRP)	Rabbit	1:100	Sigma C8198
Neuronal Nitric Oxide Synthase (nNOS)	Rabbit	1:100	Cell Signal C7D7
Nerve Growth Factor (NGF)	Rabbit	1:500	Alomone AN-240
Protein gene product 9.5 (PGP9.5)	Rabbit	1:250	Millipore AB1761-I
Substance P	Rat	1:500	BD Pharmigen 556312
Transient Receptor Potential Vanilloid 1 (TRPV1)	Rabbit	1:200	Alomone ACC-030
Tyrosine Hydroxylase (TH)	Rabbit	1:500	Pel Freez P40101
Secondary Antibodies			
Alexa Fluor 488	Goat anti-rabbit	1:1000	ThermoFisher
Alexa Fluor 555	Goat anti-rat	1:1000	ThermoFisher
Alexa Fluor 555	Goat anti-rabbit	1:1000	ThermoFisher
Alexa Fluor 555	Donkey anti-goat	1:1000	ThermoFisher
Alexa Fluor 647	Goat anti-rabbit	1:1000	ThermoFisher
Biotinylated anti-rabbit IgG	Goat anti-rabbit	1:400	Vector

CHAPTER 3. Maternal allergen exposure increases offspring lung sensory innervation and causes airway hyperreactivity

A. Abstract

Asthma is characterized by airway hyperreactivity, an abnormal tendency for airways to constrict. Maternal asthma increases the risk of childhood asthma, more so than paternal asthma, suggesting intrauterine exposures contribute to airway hyperreactivity. Here we show that pregnant mice exposed to house dust mite, and pregnant transgenic mice that overexpress interleukin-5 (IL-5), give birth to offspring with markedly increased airway sensory innervation, long lasting increases in airway neurotrophins, and airway hyperreactivity without changes in Th2 cytokines. Our results demonstrate that fetal exposure to maternal asthma or increased IL-5 contributes to developmental origins of airway hyperreactivity.

B. Introduction

Asthma affects 8.4% of US children and is characterized by airway inflammation, airway hyperreactivity, and structural remodeling of the lung. Parental asthma increases infant airway reactivity[92] and the risk of their child developing asthma[83,84]. Genetic inheritance of risk alleles and shared environments explain only part of this risk since maternal asthma imposes a greater risk for childhood asthma than paternal disease[85]. Airway reactivity at birth is already variable[90], suggesting a role for prenatal exposures in *in utero* programming of airway hyperreactivity.

Airway nerves mediate airway hyperreactivity through a structurally complex pathway. Afferent epithelial sensory nerves with cell bodies in the nodose-jugular ganglia of the vagus nerve respond to inhaled stimuli, synapse on central nervous system circuits, and trigger activation of efferent parasympathetic nerves also contained within the vagus. These efferent nerves activate parasympathetic ganglia in the airways, releasing acetylcholine, which contracts airway smooth muscle by binding to M3 muscarinic receptors. Acetylcholine also activates pre-synaptic inhibitory M2 muscarinic receptors located on efferent parasympathetic nerves to limit further acetylcholine release[160]. Blocking this reflex with muscarinic antagonists, including tiotropium, improves lung function in adults[182,577] and children with asthma[578], demonstrating the importance of airway innervation to asthma.

Woman with asthma who are intensively managed during pregnancy using fraction of exhaled nitric oxide guided therapy, a marker of type-2 inflammation, give birth to infants with reduced risk of asthma compared to children born to mothers who received symptom-only directed care[89]. The ratio between type-2 and type-1 cytokines is associated with childhood asthma in human pregnancies[579] and treating allergen exposed murine mothers with interferon-Y[126] or blocking maternal IL-4[120] reduces offspring airway hyperreactivity. These data suggest that type 2 cytokines may influence *in utero* development of offspring airway hyperreactivity. Many patients with asthma have eosinophil-

predominant inflammation mediated by elevated interleukin-5, a cytokine necessary for eosinophil hematopoiesis, survival, and recruitment. Eosinophils are actively recruited to airway nerves[372] and there are increased nerve-associated eosinophils in lungs of patients who died from asthma and after allergen-challenge in animals[373]. Eosinophils promote nerve growth[207] and secrete major basic protein, which inhibits M2 muscarinic receptor function[42,375], leading us to question whether maternal type-2 inflammation affects fetal airway nerve development. Here we tested whether maternal house dust mite (HDM) exposure or IL-5 increases airway sensory innervation in offspring to contribute to airway hyperreactivity.

C. Results

C1. Allergen exposure during pregnancy increased airway inflammation but not airway hyperreactivity in mothers

I exposed female mice to inhaled HDM or vehicle daily for 4 weeks before and then for 3 weeks throughout pregnancy (Figure 3a). Reflex bronchoconstriction in response to inhaled serotonin was the same between vehicle and HDM exposed mothers after eight weeks of treatment (Figure 3b). Airway inflammation increased in HDM exposed mothers (Figure 3c) compared to vehicle-exposed mothers and consisted mostly of activated, vacuolated macrophages (Figure 3e; quiescent airway macrophages in vehicle exposed mothers Figure 3d).

C2. Maternal HDM increased offspring airway hyperreactivity and inflammation.

I exposed female mice to inhaled HDM daily for 4 weeks before pregnancy and an additional 3 weeks throughout pregnancy (Figure 3a). When offspring reached adulthood (≥8 weeks), I sedated, mechanically ventilated and exposed them to inhaled serotonin to induce reflex bronchoconstriction. I chose serotonin because it causes consistent, reproducible bronchoconstriction in mice[549] via activation of vagal sensory nerves[179]. Baseline airway resistance before serotonin was not different between offspring of mothers exposed to vehicle vs. HDM. Untreated offspring had negligible bronchoconstriction after inhaling serotonin (Figure 4b) and sensitization and exposure to vehicle did not alter these responses (Figure 4c). I included both untreated and vehicle exposed animals since inhaled anesthetics used during vehicle and HDM exposures activate sensory nerve ion channels[569,570], affect tachykinin physiology[571], and mask neuron chemosensitivity[580]. However, since there was no difference in airway physiology, only vehicle treated offspring were included in further studies. HDM sensitization and challenge (as described in Figure 4a) caused airway hyperreactivity in all offspring (Figure 4d), however, airway hyperreactivity was much greater in offspring born to HDM exposed mothers than in offspring born to vehicle-exposed mothers (Figure 4d).

Maternal HDM exposure during pregnancy is known to increase airway inflammation and Th2 cytokines after HDM challenge of their offspring[581]. In my study, there was no difference in lavage total inflammatory cells, eosinophils,

macrophages, neutrophils (Figure 4e-h), or lymphocytes (none detected) between vehicle treated offspring from mothers exposed to HDM compared with vehicle. However, after HDM sensitization and challenge, offspring from HDM exposed mothers had significantly more inflammatory cells in their airways than offspring from vehicle-exposed mothers. Augmented inflammatory cell counts and airway hyperreactivity occurred independent of changes in IL-5 and IL-13, as both cytokines were similar in offspring from HDM vs. vehicle-exposed mothers (Figure 4i-j). However, HDM exposure increased lavage eotaxin, an eosinophil-specific chemoattractant, more in offspring from HDM exposed mother than offspring from vehicle-exposed mothers (Figure 4k).

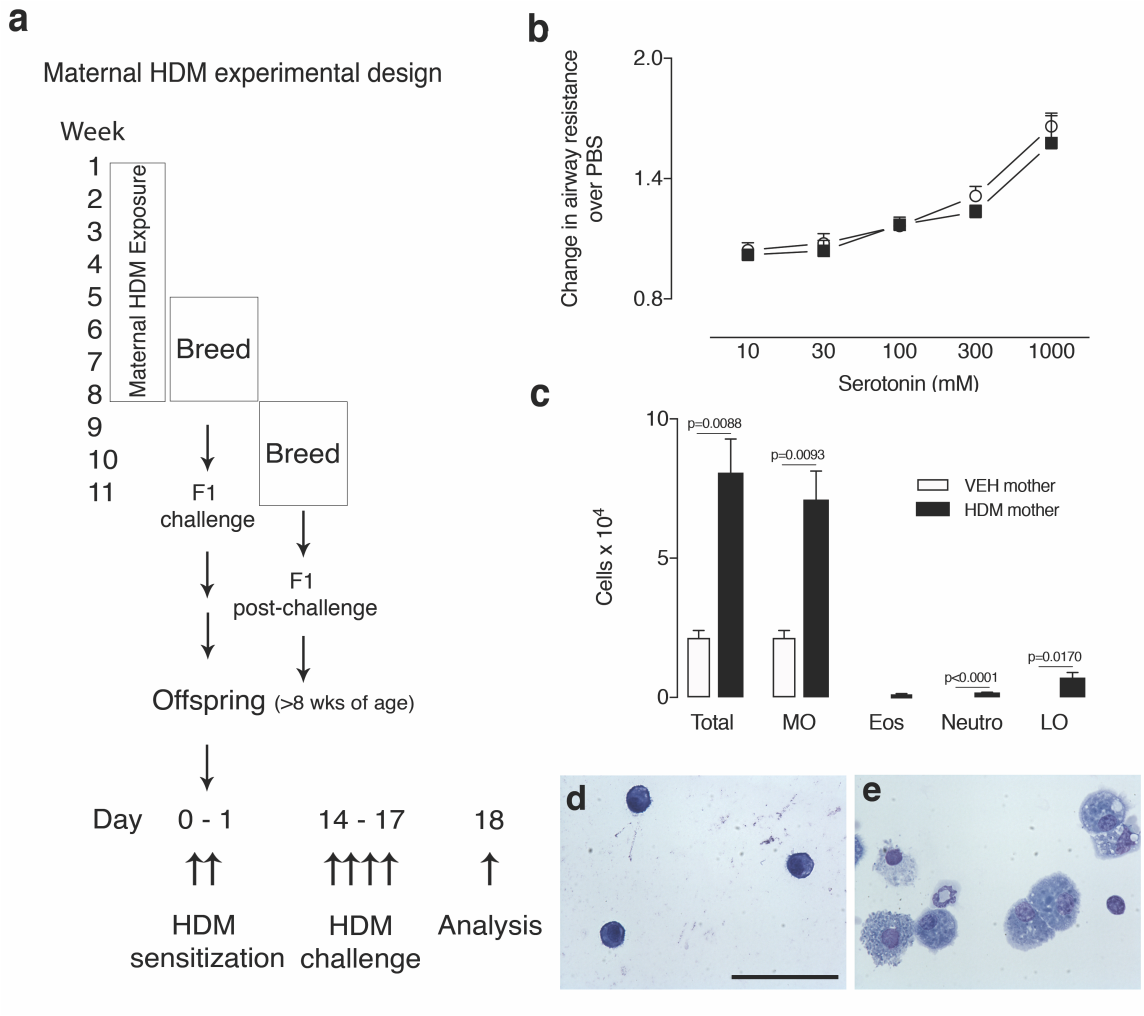
a
Maternal HDM experimental design
Week
1
2
3
4
5
6
7
8
9
10
11
Maternal HDM Exposure
Breed
Breed
F1
challenge
F1
post-challenge
Offspring (>8 wks of age)
Day
0 - 1
14 - 17
18
HDM
sensitization
HDM
challenge
Analysis
b
Change in airway resistance
over PBS
2.0
1.4
0.8
10
30
100
300
1000
Serotonin (mM)
c
Cells x 10^4
10
5
0
p=0.0088
p=0.0093
p<0.0001
p=0.0170
VEH mother
HDM mother
Total
MO
Eos
Neutro
LO
d
e

Figure 3. HDM exposure during pregnancy increased airway inflammation but not airway hyperreactivity. (a) Female mice were exposed to HDM or vehicle intranasally for 4 weeks before and then for 3 weeks during pregnancy. Exposure stopped once pups were born. Breeding continued in the absence of HDM exposure and mice gave birth to a second litter (F1 post-challenge). (a) Maternal airway reactivity was tested in mice that failed to become pregnant or that killed their pups shortly after birth. Reflex bronchoconstriction in response to inhaled serotonin was the same between vehicle (◯, n=3) and HDM exposed mothers (■, n=3) after 8 weeks of treatment. (b) Airway total cells, macrophages, neutrophils, lymphocytes, but not eosinophils, increased in mothers exposed to HDM compared to vehicle. (c) Alveolar macrophages looked normal in vehicle exposed mothers (d) compared to vacuolated, enlarged macrophages in HDM exposed mothers (e). Scale bar = 75 μM. Data graphed as mean ± SEM. P values represent two-tailed t-test.

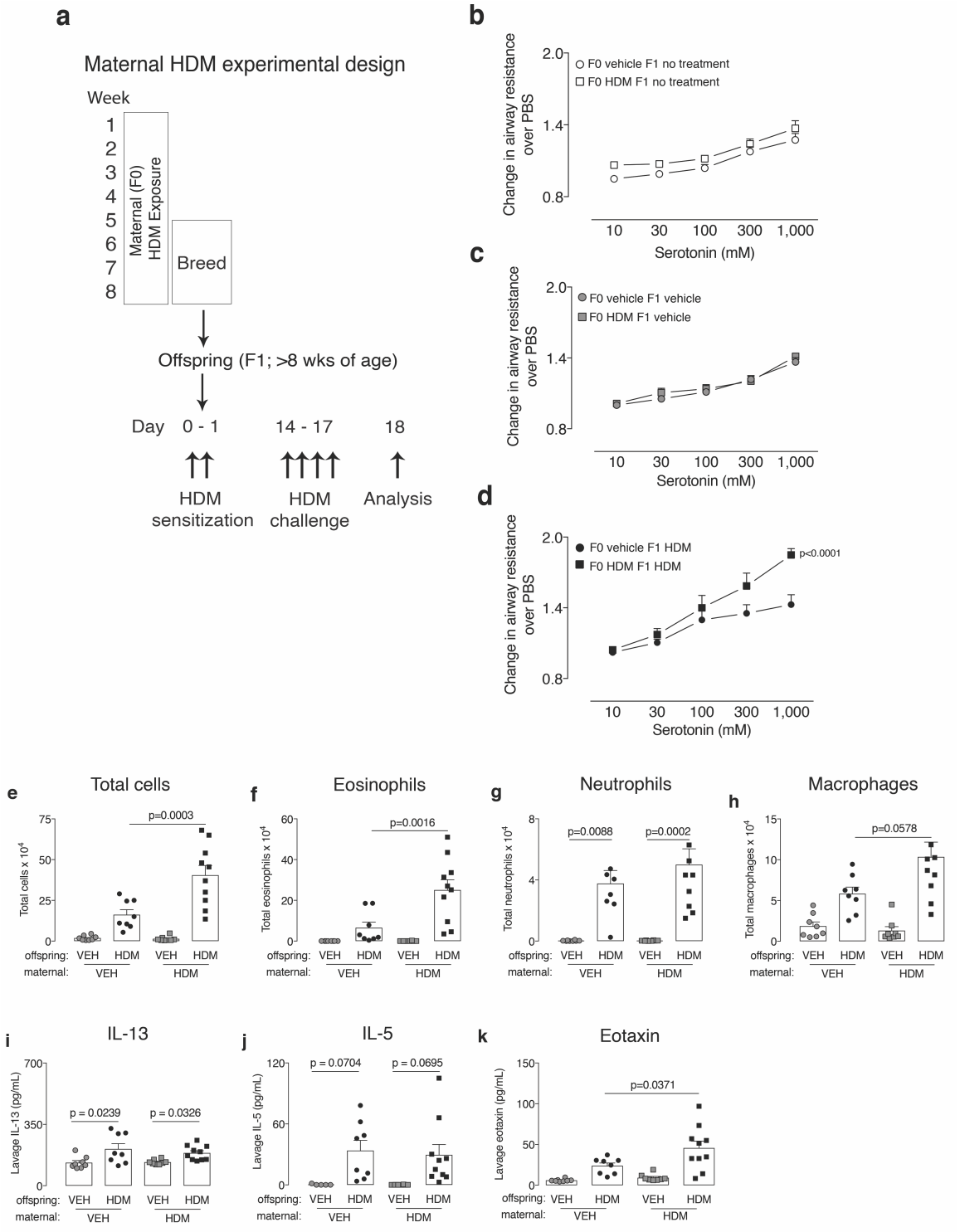
a
Maternal HDM experimental design
Week
1
2
3
4
5
6
7
8
Maternal (F0) HDM Exposure
Breed
Offspring (F1; >8 wks of age)
Day 0 - 1
14 - 17
18
HDM sensitization
HDM challenge
Analysis
b
F0 vehicle F1 no treatment
F0 HDM F1 no treatment
Change in airway resistance over PBS
Serotonin (mM)
c
F0 vehicle F1 vehicle
F0 HDM F1 vehicle
Change in airway resistance over PBS
Serotonin (mM)
d
F0 vehicle F1 HDM
F0 HDM F1 HDM
p<0.0001
Change in airway resistance over PBS
Serotonin (mM)
e
Total cells
Total cells x 10^4
p=0.0003
f
Eosinophils
Total eosinophils x 10^4
p=0.0016
g
Neutrophils
Total neutrophils x 10^4
p=0.0088
p=0.0002
h
Macrophages
Total macrophages x 10^4
p=0.0578
offspring: VEH HDM VEH HDM
maternal: VEH HDM
i
IL-13
Lavage IL-13 (pg/mL)
p = 0.0239
p = 0.0326
j
IL-5
Lavage IL-5 (pg/mL)
p = 0.0704
p = 0.0695
k
Eotaxin
Lavage eotaxin (pg/mL)
p=0.0371

Figure 4. Maternal HDM exposure increased offspring airway hyperreactivity. (a) Female mice were exposed to HDM (intranasal) for 4 weeks before and then throughout pregnancy. Once offspring reach adulthood, physiology was measured at baseline (b), after vehicle (c) and HDM exposure (d). Serotonin caused minimal reflex bronchoconstriction in offspring at baseline (b, ○=vehicle-exposed mothers, n=8; □=HDM exposed mother, n=8) or after vehicle sensitization and exposure (c, ●=vehicle-exposed offspring from vehicle-exposed mothers, n=8; ■= vehicle-exposed offspring from HDM exposed mothers, n=8). Maternal HDM exposure potentiated airway hyperreactivity in HDM exposed offspring (d, ■, n=10) compared with HDM exposed offspring from vehicle-exposed mothers (d, ●, n=8, $p<0.0001$, df=4, F=8.164). Maternal HDM exposure increased offspring lavage total cells (e, $p=0.0003$, $t=4.912$, df=26), eosinophils (f, $p=0.0016$, $t=4.122$, df=30), and macrophages (g, $p=0.0578$, df=30, $t=2.755$), but not neutrophils (h), IL-13 (i), or IL-5 (j), after offspring HDM exposure. Lavage eotaxin increased in all offspring after HDM exposure, but more in offspring from HDM exposed mothers than in offspring from vehicle exposed mothers (k, $p=0.0371$, $t=2.939$, df=30). Data presented as mean ± SEM. P values represent interaction term of repeated measure two-way ANOVA for physiology data or Sidak post-test for lavage inflammatory cells and cytokines. HDM treatment significantly increased all inflammatory cells and cytokines, however P value not shown for clarity when maternal HDM exposure had significant effect.

C3. Maternal HDM exposure increased offspring airway innervation.

I next assessed epithelial sensory nerve structure and parasympathetic ganglia number in whole-mount, optically cleared tracheas stained with substance P (a sensory nerve marker) and PGP9.5 (a nonspecific, pan neuronal maker), using confocal imaging and three-dimensional nerve models[227] (Figure 5a-h). I chose to model tracheal innervation because sensory epithelial innervation is greatest in the trachea, diminishes rapidly, and is rare to nonexistent beyond secondary bronchi in mice[227]. Furthermore, stimulation of upper airway nerves initiates reflex bronchoconstriction to contract distal bronchioles. Imaging the trachea also permitted me to capture both parasympathetic ganglia and epithelial nerves in the same tissue. Epithelial sensory nerves in offspring from HDM exposed mothers were structurally more complex, with increased nerve length and branching (Figure 5i-j), compared with offspring from vehicle-exposed mothers. Parasympathetic ganglia were not structurally different between offspring (number of ganglia from offspring from vehicle mothers, 94±4 vs from offspring from HDM mothers, 97±4), suggesting maternal HDM exposure largely affected sensory nerve development.

Neurotrophins increase airway innervation[6,503,535,582], and are elevated in patients with asthma[415]. I measured four neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell derived neurotrophic factor (GDNF), and neurotrophin-4 (NT-4) in lung lavage. BDNF (Figure 5k), but not NGF,

GDNF, or NT4 (Figure 5 l-n), was elevated in offspring from HDM exposed mothers compared with offspring from vehicle-exposed mothers.

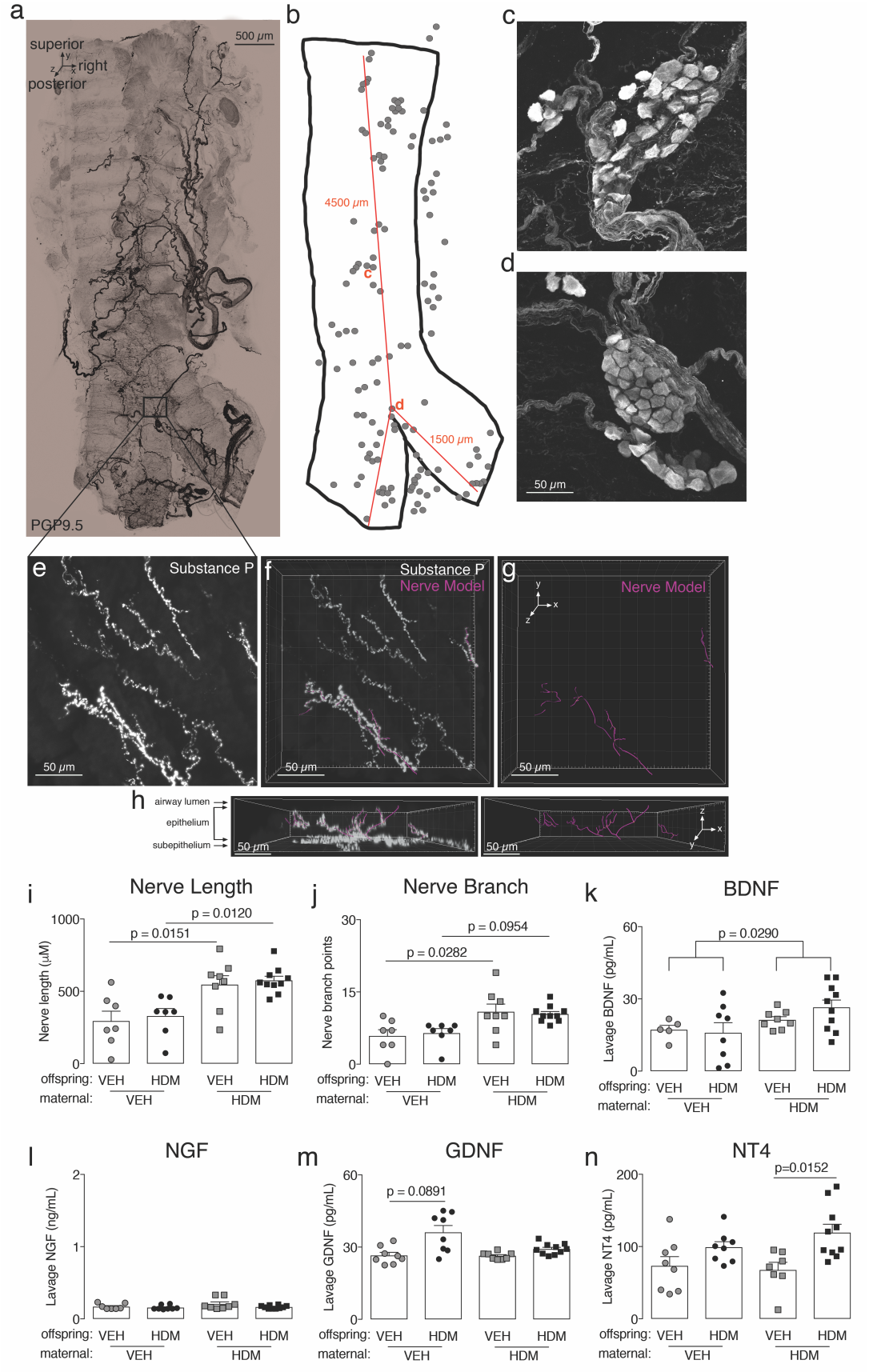
a
superior
right
posterior
500 μm
PGP9.5
b
4500 μm
c
d
1500 μm
c
d
50 μm
e
Substance P
50 μm
f
Substance P
Nerve Model
50 μm
g
Nerve Model
50 μm
h
airway lumen
epithelium
subepithelium
50 μm
50 μm
i
Nerve Length
Nerve length (μM)
p = 0.0120
p = 0.0151
offspring: VEH HDM VEH HDM
maternal: VEH HDM
j
Nerve Branch
Nerve branch points
p = 0.0954
p = 0.0282
offspring: VEH HDM VEH HDM
maternal: VEH HDM
k
BDNF
Lavage BDNF (pg/mL)
p = 0.0290
offspring: VEH HDM VEH HDM
maternal: VEH HDM
l
NGF
Lavage NGF (ng/mL)
offspring: VEH HDM VEH HDM
maternal: VEH HDM
m
GDNF
Lavage GDNF (pg/mL)
p = 0.0891
offspring: VEH HDM VEH HDM
maternal: VEH HDM
n
NT4
Lavage NT4 (pg/mL)
p=0.0152
offspring: VEH HDM VEH HDM
maternal: VEH HDM

Figure 5. Maternal HDM exposure increased offspring airway sensory innervation. Whole trachea were stained with substance P and PGP9.5 antibodies, optically cleared, and imaged with laser scanning microscope (20x, 0.8 numerical aperture). Trachea z-stacks were compressed into maximum intensity projections (a; black and white inverted to better highlight nerves) and ganglia maps constructed for each trachea (b; each circle identifies location of one ganglia). Higher magnification image (63x, 1.4 numerical aperture) of ganglia 'C' (c) and 'D' (d) denoted in panel b. Three 63x images per animal were then taken in the midline carina, extending from the serosal surface through airway lumen. Substance P positive epithelial and subepithelial nerves in optically cleared, whole mount trachea. 63x, maximum intensity projection of z-stack confocal image (e). 3D model of epithelial nerves with (f) and without (g) fluorescent, full z-stack image oriented with line of site in z-plane. Same image rotated around x-axis demonstrates epithelial location of nerve models (h). Maternal HDM exposure increased offspring airway sensory nerve length (h, $p=0.0151$, $t=3.317$, $df=28$ and $p=0.0120$, $t=3.406$, $df=28$), branching (I, $p=0.0282$, $t=3.067$, $df=28$), and lavage BDNF (k, $p=0.0290$, $F=5.319$) compared to offspring from vehicle-exposed mothers. Maternal HDM exposure did not change offspring lavage NGF (l), GDNF (m), or NT4 (n). Data presented as mean ± SEM. BDNF P value represents main effect of maternal treatment after two-way ANOVA. Other P values represent Sidak post-test after a two-way ANOVA. N=8 vehicle and n=8 HDM exposed offspring from vehicle exposed mothers. N=8 vehicle and n=10 HDM exposed offspring from HDM exposed mothers, except n=7 for offspring

from vehicle exposed mothers for nerve length and branch points. One vehicle treated offspring from HDM-exposed mother had insufficient lavage fluid to run NT4 in duplicate, thus n=7.

C4. Maternal allergen exposure increases fetal innate immune cells

To test whether maternal HDM exposure elicited a fetal immune response, I collected fetal blood between gestation days 18-20 of offspring from HDM and vehicle-exposed mothers. In fetal blood smears, neutrophils, eosinophils, basophilic cells (Figure 6a-c), and lymphocytes (remaining percentage) were counted. Basophilic cells comprised several types of immune cells, likely mast cell progenitors and basophils, but were difficult to distinguish without cell surface markers and thus combined. Maternal HDM exposure increased fetal eosinophils, basophilic cells, and neutrophils compared to fetuses from vehicle-exposed mother (Figure 6a-c).

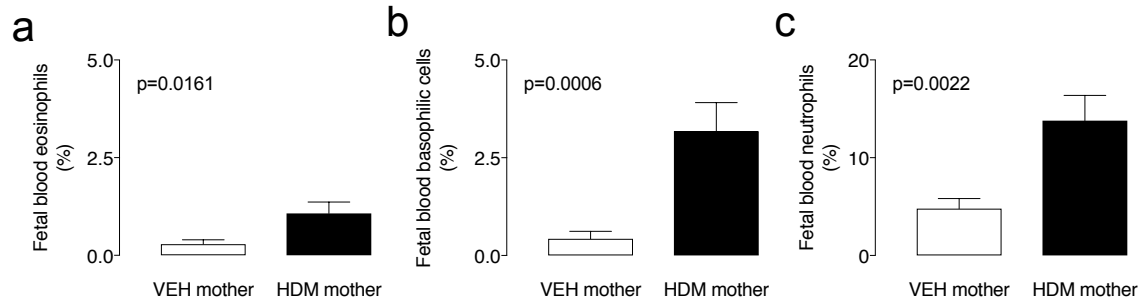

Figure 6. Maternal HDM exposure increased circulating, fetal innate immune cells. Eosinophils (a), basophilic cells (b), and neutrophils (c) were increased in fetal blood from HDM exposed mothers (n=28 fetuses, n=3 pregnancies) compared with vehicle exposed mothers (n=28 fetuses, n=3 pregnancies).

C4. Maternal effect on offspring airway hyperreactivity persists into second pregnancies and second generation without continued allergen exposure.

Airway inflammation due to allergen exposure resolves after 4 weeks in mice[7,583]. Thus, I tested whether active allergen exposure was required for airway hyperreactivity in offspring or if sustained maternal inflammation after allergen exposure could increase airway hyperreactivity of offspring. Mothers that were previously exposed to HDM or vehicle became pregnant with a second litter (as described in Figure 3a) and offspring (F1 post-challenge) airway responses measured. Sensitization and challenge with HDM caused greater airway hyperreactivity in offspring born to mothers exposed to HDM before pregnancy than in offspring born to mothers exposed to vehicle before pregnancy (Figure 7a). Airway hyperreactivity was completely blocked by the muscarinic antagonist atropine (Figure 7b), demonstrating that a vagal reflex mediated serotonin-induced bronchoconstriction. I tested nerve reflexes in offspring born to mothers previously challenged with allergen by surgically cutting the vagus nerves. Vagotomy markedly worsened airway hyperreactivity in offspring from mothers exposed to vehicle before pregnancy (Figure 7c), but suppressed airway hyperreactivity in offspring from mothers exposed to HDM before pregnancy (Figure 7d). These data demonstrate that persistent maternal inflammation in the absence of continued allergen exposure increases offspring airway hyperreactivity.

To test if maternal allergen exposure increased offspring airway hyperreactivity in subsequent generations, allergen-naïve first generation (F1) mice that were developing during maternal (F0) HDM exposure were bred together to produce second-generation (F2) mice. Once F2 mice reached adulthood, they were allergen sensitized and challenged and airway responses to inhaled serotonin measured. F2 mice descended from HDM-exposed mothers had greater airway hyperreactivity than F2 mice descended from vehicle-exposed mothers (Figure 7e). Bronchoconstriction was so severe in F2 mice from HDM-exposed mothers that only 3 mice survived the entire dose-response curve. These data demonstrate that maternal allergen exposure causes offspring airway hyperreactivity that is heritable across generations, likely via epigenetic changes.

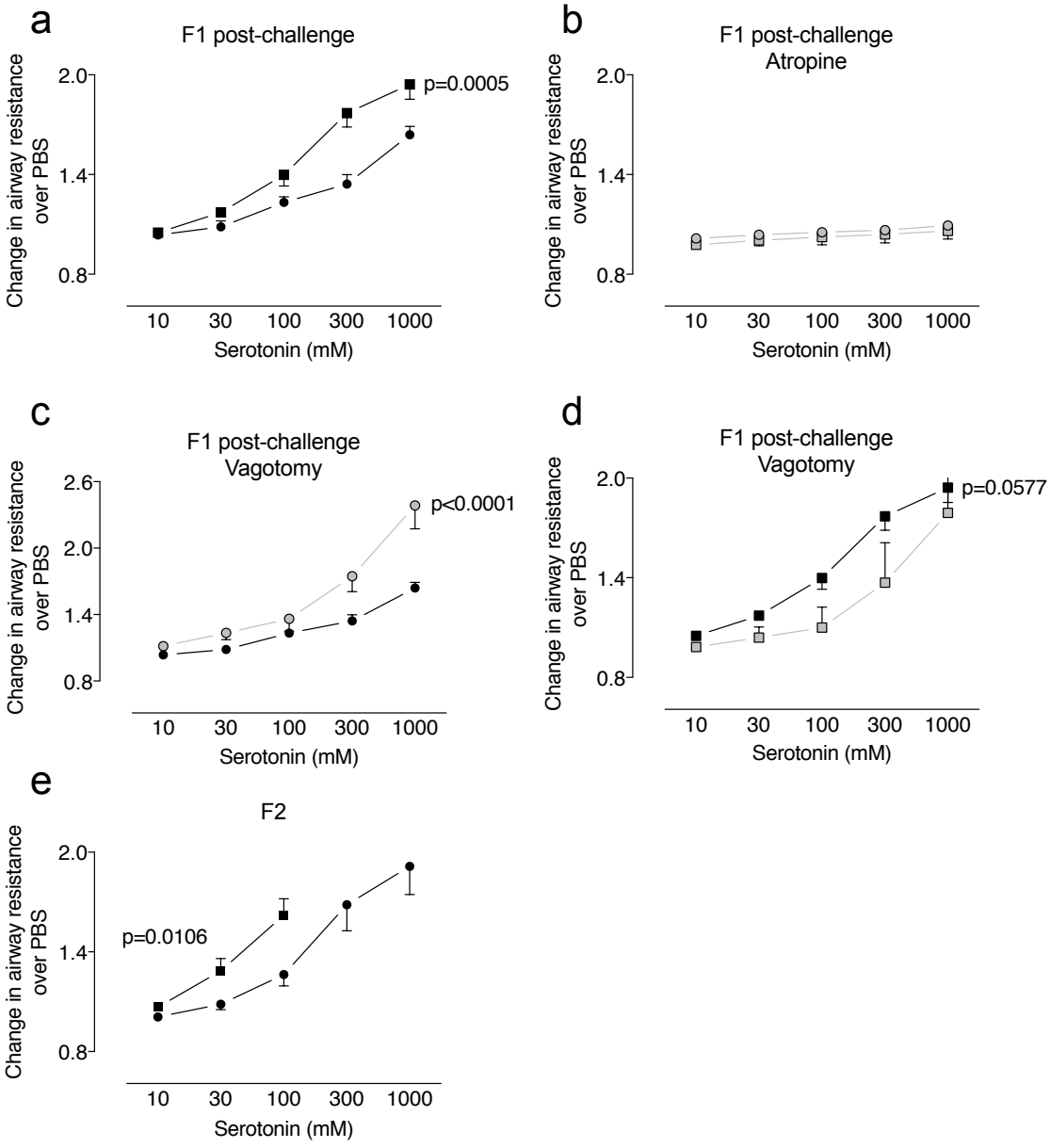
a
F1 post-challenge
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0005
10
30
100
300
1000
Serotonin (mM)
b
F1 post-challenge
Atropine
Change in airway resistance over PBS
2.0
1.4
0.8
10
30
100
300
1000
Serotonin (mM)
c
F1 post-challenge
Vagotomy
Change in airway resistance over PBS
2.6
2.0
1.4
0.8
p<0.0001
10
30
100
300
1000
Serotonin (mM)
d
F1 post-challenge
Vagotomy
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0577
10
30
100
300
1000
Serotonin (mM)
e
F2
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0106
10
30
100
300
1000
Serotonin (mM)

Figure 7. Maternal HDM exposure increased offspring airway hyperreactivity in second pregnancies and second generations. Female mice were challenged with HDM or vehicle during their first pregnancy (offspring denoted F1) and then became pregnant again (offspring denoted F1 post-challenge). Mothers were not exposed to HDM or vehicle during their second pregnancy. (a) Offspring born to mothers exposed to HDM before pregnancy were hyperreactive (black squares, n=10) compared to offspring born to mothers exposed to vehicle before pregnancy (black circles, n=10). (b) Atropine blocked airway hyperreactivity in all offspring regardless of prior maternal exposure (n=3 offspring from mothers exposed to HDM before pregnancy, n=4 offspring from mothers exposed to vehicle before pregnancy). (c) Vagotomy (grey circles, n=6) worsened airway hyperreactivity in HDM challenged offspring born to mothers exposed to vehicle before pregnancy. (d) Vagotomy (grey squares, n=3) reduced airway hyperreactivity in HDM challenged offspring born to mothers exposed to HDM before pregnancy. Offspring (F1) from vehicle and HDM-exposed mothers (F0) were bred to generate second generation (F2) offspring. F1 mice were not exposed to HDM or vehicle during pregnancy. (e) Increased airway hyperreactivity persisted in F2 mice descended from HDM-exposed F0 mothers (e).

C4. Maternal interleukin-5 increases offspring airway hyperreactivity and inflammation

I next tested whether increased maternal IL-5 could increase offspring airway innervation and promote offspring airway hyperreactivity. I utilized an interleukin-5 transgenic (IL5tg)[37] mouse that expresses IL-5 in lung epithelial cells to independently test whether elevated maternal IL-5 caused offspring airway hyperreactivity.

I bred hemizygous IL5tg female mice with wild-type (WT) male mice to produce WT offspring exposed to maternal IL-5 *in utero* (Figure 8a). Once WT offspring reached adulthood, they were mechanically ventilated and exposed to inhaled serotonin to induce reflex bronchoconstriction. WT offspring from IL5tg mothers had increased reflex bronchoconstriction compared to offspring born to WT mothers (Figure 8b). Sensitization and exposure to vehicle suppressed reflex bronchoconstriction in both groups (Figure 8c), thus both baseline and vehicle-control mice were included in subsequent experiments. Sensitization and challenge with HDM caused much greater airway hyperreactivity in offspring born to IL5tg mothers than in offspring born to WT mothers (Figure 8d). Bronchoconstriction was so severe in HDM exposed WT offspring from IL5tg mothers that only one of eight offspring survived the full dose response curve.

At baseline, there was no difference in lavage total inflammatory cells, eosinophils, neutrophils, macrophages, (Figure 8e-h), or lymphocytes (none

detected) between offspring of mothers with different genotypes. However, after HDM sensitization and exposure, offspring from IL5tg mothers had significantly more eosinophils, neutrophils, and macrophages in their airways than HDM exposed offspring from WT mothers (Figure 8e-h). Augmented inflammatory cell counts again occurred independent of changes in Th2 cytokines, as IL-5, IL-13, and eotaxin (Figure 8i-k) were similar in offspring from mothers with different genotypes.

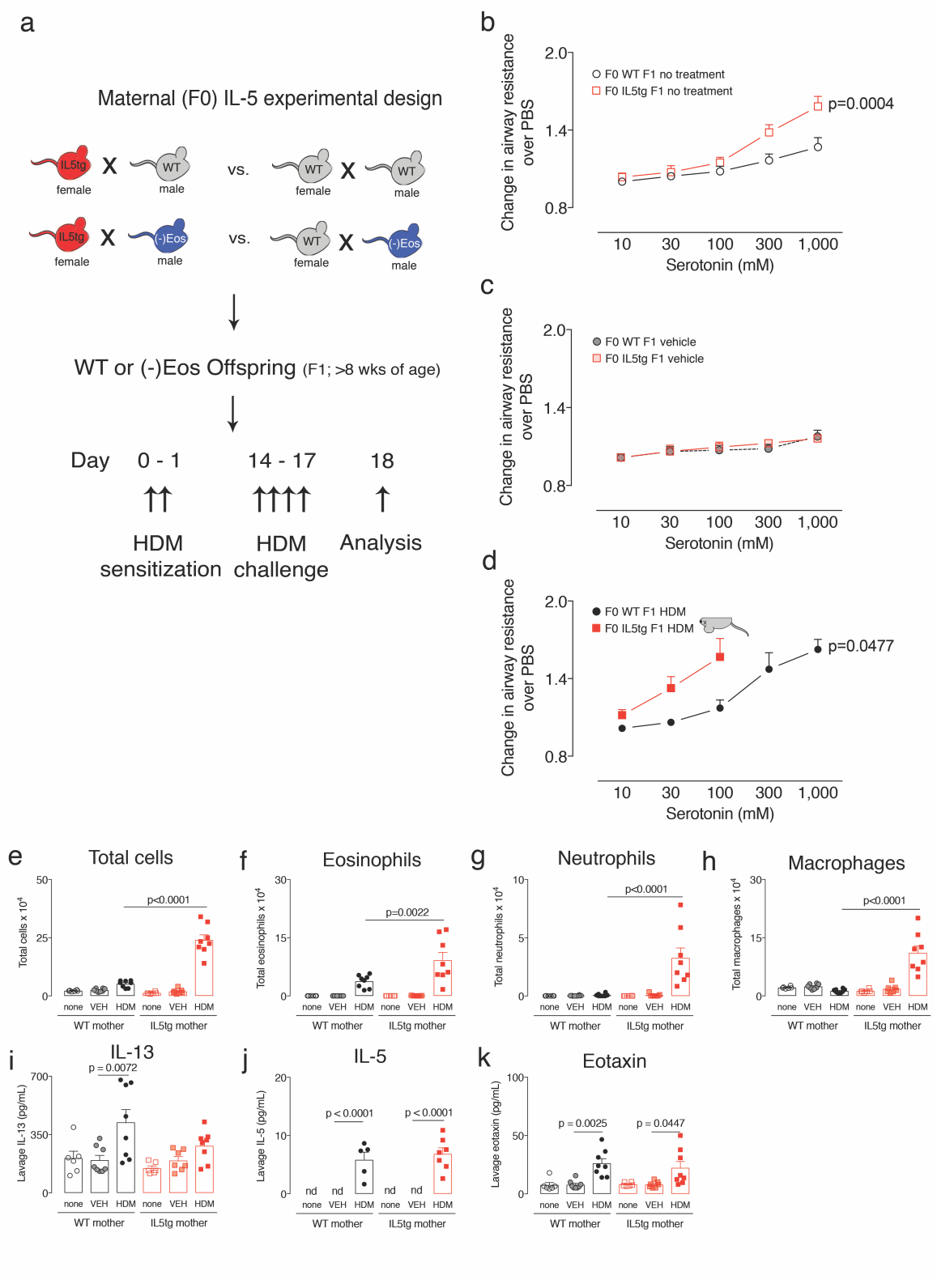
a
Maternal (F0) IL-5 experimental design
IL5tg
female
x
WT
male
vs.
WT
female
x
WT
male
IL5tg
female
x
(-)Eos
male
vs.
WT
female
x
(-)Eos
male
WT or (-)Eos Offspring (F1; >8 wks of age)
Day 0 - 1 14 - 17 18
HDM sensitization
HDM challenge
Analysis
b
Change in airway resistance over PBS
F0 WT F1 no treatment
F0 IL5tg F1 no treatment
p=0.0004
Serotonin (mM)
c
F0 WT F1 vehicle
F0 IL5tg F1 vehicle
d
F0 WT F1 HDM
F0 IL5tg F1 HDM
p=0.0477
e
Total cells
Total cells x 10^4
p<0.0001
f
Eosinophils
Total eosinophils x 10^4
p=0.0022
g
Neutrophils
Total neutrophils x 10^4
p<0.0001
h
Macrophages
Total macrophages x 10^4
p<0.0001
none VEH HDM none VEH HDM
WT mother
IL5tg mother
i
IL-13
Lavage IL-13 (pg/mL)
p = 0.0072
j
IL-5
Lavage IL-5 (pg/mL)
p < 0.0001
p < 0.0001
nd
k
Eotaxin
Lavage eotaxin (pg/mL)
p = 0.0025
p = 0.0447

Figure 8. Maternal IL-5 increased offspring airway hyperreactivity. Design of elevated maternal IL-5 experiments. IL5tg = IL-5 transgenic mice. (-)Eos = congenitally eosinophil deficient mice. (a). Exposure to maternal IL-5 increased baseline reflex bronchoconstriction in WT offspring from IL5tg mothers (b; □, n=6) compared with WT offspring from WT mothers (○, n=6, p=0.0004, F=6.559, df=4). Exposure to maternal IL-5 potentiated HDM-induced airway hyperreactivity in WT offspring from IL5tg mothers (d; ■, n=8) compared with WT offspring from WT mothers. (d, ●, n=8, p=0.0477, F=3.398, df=2) (vehicle control in figure c; □ = vehicle exposed WT offspring from IL5tg mothers, n=7;○=vehicle exposed WT offspring from WT mothers, n=8). Maternal IL-5 increased offspring lavage total cells (e, p<0.0001, t=13.05, df=38), eosinophils (f, p=0.0018, t=4.289, df=38), macrophages (g, p<0.0001, t=8.736, df=38), and neutrophils (h, p<0.0001, t=6.124, df=38), but did not increase lavage IL-13 (i), IL-5 (j), or eotaxin (k) after HDM exposure. Three HDM exposed offspring from WT mothers had insufficient lavage fluid to measure IL-5 in replicate, thus n=5. Data presented as mean ± SEM. P values for physiology represent interaction term of repeated measure two-way ANOVA and Sidak post-test comparisons for inflammatory cells and cytokines. Statistics for figure d calculated only for 10-100 mM serotonin doses (see methods for further clarification). HDM significantly increased all inflammatory cells, however P values omitted for clarity where maternal genotype had effect. nd = not detected.

C5. Maternal IL-5 increased offspring airway innervation

Similar to offspring from HDM exposed mothers, WT offspring from IL5tg mothers had increased epithelial sensory innervation, in both substance P expressing and total epithelial nerves populations, compared to WT offspring from WT mothers (Figure 9a-d) and again there was no difference in number of parasympathetic ganglia (WT mother, 84±4; IL5tg mother, 89±6).

In contrast to the HDM exposure model, WT offspring from IL5tg mothers had elevated lavage NGF, in both airway lavage (Figure 9f) and airway epithelium (Figure 10) compared with WT offspring from WT mothers, while there was no difference in lavage BDNF (Figure 9e), GDNF (Figure 9g), or NT4 (Figure 9h). To test whether increased nerve complexity represented structural changes in individual neurons versus more neurons innervating the airway epithelium, airway-specific neurons were retrograde labeled with inhaled fluorescent wheat germ agglutinin and counted (Figure 9i-j). The number of vagal nodose-jugular neurons innervating airway epithelium was the same in offspring from mothers with different genotypes (Figure 9k), demonstrating that increased nerve length and branching is due to structurally more complex neurons rather than increased cells in the ganglia.

In addition to increasing nerve complexity, maternal IL-5 increased substance P in lavage and in epithelial nerves at baseline (Figure 11a- b). In lavage, HDM exposure additionally increased substance P in offspring from both WT and IL5tg

mothers. In contrast, in epithelial nerves, substance P increased following HDM exposure only in offspring from WT mothers suggesting that neuronal substance P was already maximal in WT offspring from IL5tg mothers. Neuronal substance P fluorescence within smooth muscle was unaffected by either maternal genotype or by HDM exposure (data not shown), suggesting the effect of maternal IL-5 on substance P expression was limited to nerves innervating the epithelium.

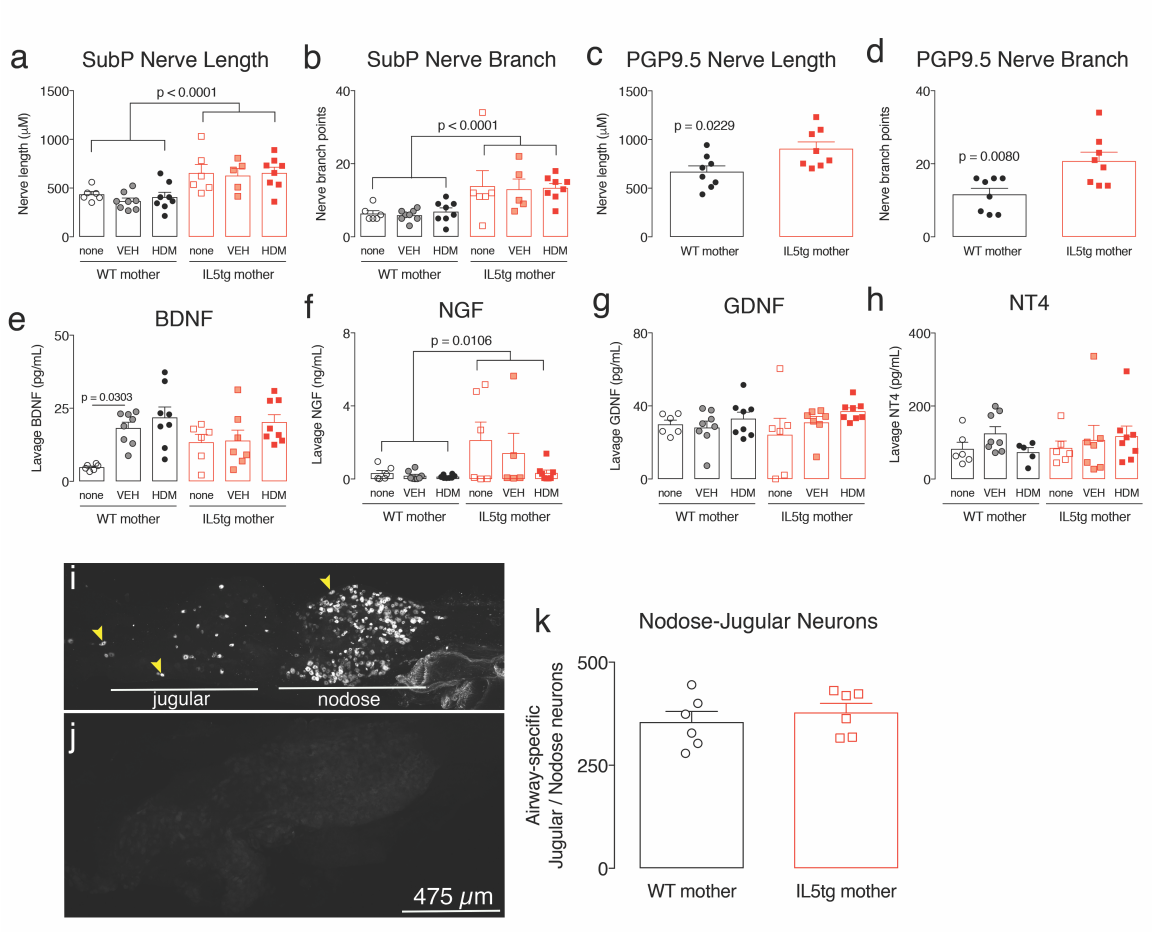
a
SubP Nerve Length
Nerve length (μM)
p < 0.0001
b
SubP Nerve Branch
Nerve branch points
p < 0.0001
c
PGP9.5 Nerve Length
Nerve length (μM)
p = 0.0229
d
PGP9.5 Nerve Branch
Nerve branch points
p = 0.0080
none VEH HDM none VEH HDM
WT mother
IL5tg mother
e
BDNF
Lavage BDNF (pg/mL)
p = 0.0303
f
NGF
Lavage NGF (ng/mL)
p = 0.0106
g
GDNF
Lavage GDNF (pg/mL)
h
NT4
Lavage NT4 (pg/mL)
i
jugular
nodose
j
475 μm
k
Nodose-Jugular Neurons
Airway-specific
Jugular / Nodose neurons
WT mother
IL5tg mother

Figure 9. Maternal IL-5 increased offspring airway sensory innervation. Maternal IL-5 increased offspring airway substance P positive nerve length (a, $p<0.0001$, $F=27.17$, $df=1$), branching (b, $p<0.0001$, $F=19.5$, $df=1$), PGP9.5 positive nerve length (c, $p=0.0229$, $t=2.555$, $df=14$) and branching (d, $p=0.0080$, $t=3.087$, $df=14$), and lavage NGF (f, $p=0.0106$, $F=7.283$, $df=1$) compared to offspring from WT mothers. Maternal IL-5 did not change offspring lavage BDNF (e), GDNF (g), or NT4 (h). Wheat-germ agglutinin tagged with Alexa Flour 555 was administered intranasally and nodose-jugular ganglia dissected free 16 hours later to identify fluorescent, retrograde labeled neurons (i; yellow arrowheads point to individual neurons). Negative control nodose-jugular ganglia from mouse that did not receive fluorescent wheat-germ agglutinin (j). Intensity maximum of (j) is decreased 3-fold compared to (i) to show tissue outline since background autofluorescence is very low. Data presented as mean ± SEM. Substance P nerve length and branch points and NGF P value represents main effect of maternal IL-5 after two-way ANOVA. PGP9.5 nerve length and branch point P value represents two-tailed unpaired t-test. BDNF P value represents Sidak post-test after a two-way ANOVA. N=6 for untreated offspring from WT and IL5tg mothers. N=8 vehicle and n=8 HDM exposed offspring from WT mothers and n=7 vehicle and n=8 HDM exposed offspring from IL5tg mothers for all measurements except as follows. Two vehicle exposed offspring from IL5tg mothers had insufficient lavage fluid to run NGF in duplicate, thus n=5. Three HDM exposed offspring from WT mothers had insufficient lavage fluid to run NT4 in duplicate, thus n=5. Two vehicle exposed offspring from IL5tg mothers had

poor quality nerve images which prevented accurate modeling, thus n=5. N=6 offspring per maternal genotype for nodose-jugular neuron counts.

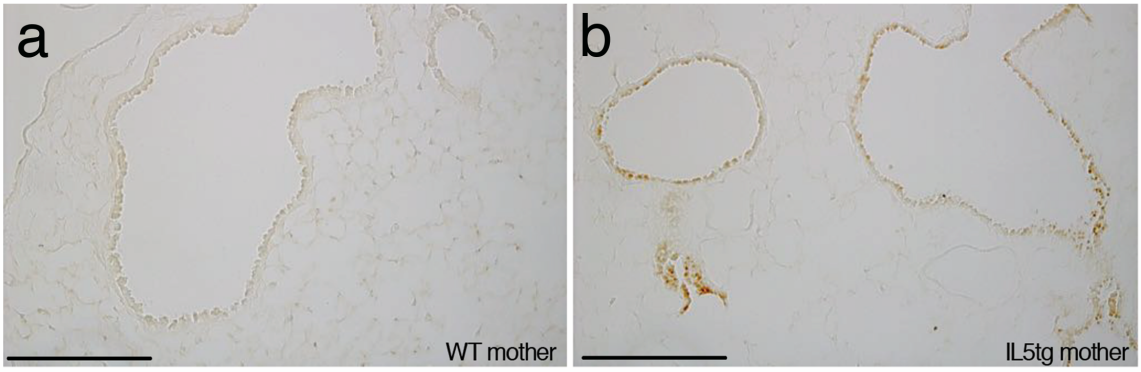

Figure 10. Maternal IL-5 increased offspring NGF. Epithelium expressed more NGF in offspring from IL5tg mothers (b) compared with offspring from WT mothers (a).

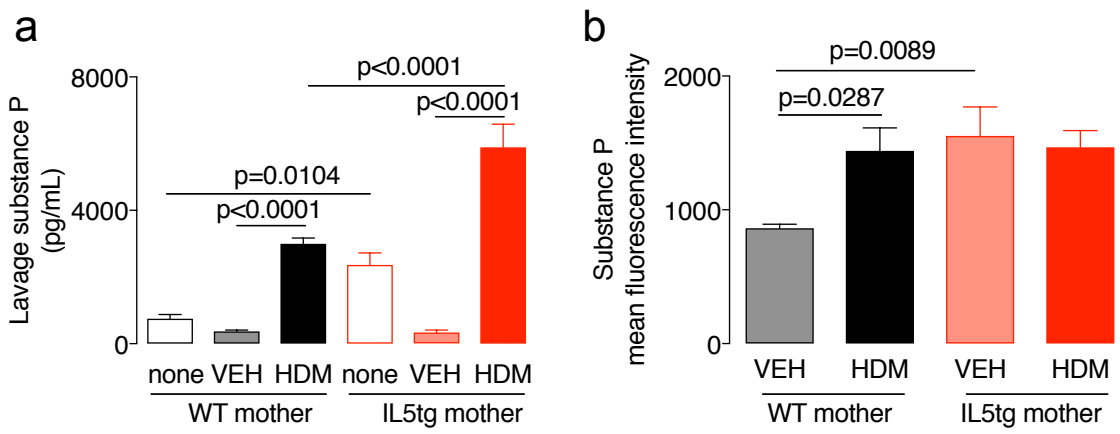

Figure 11. Maternal IL-5 increased offspring lavage and epithelial nerve substance P. (a) Offspring from IL5tg mothers (n=6) had increased lavage substance P at baseline compared with offspring from WT mothers (n=8). Isoflurane sedation and vehicle exposure suppressed lavage substance P in both groups (offspring fro IL5tg mother, n=5; offspring from WT mother, n=8). HDM exposure increased lavage substance P in both groups, but to a greater extent in offspring from IL5tg mothers (n=8) compared with offspring from WT mothers (n=8). (b) Offspring from IL5tg mothers had increased nerve substance P before HDM exposure (n=6) compared with offspring from WT mothers (n=6). HDM exposure increased nerve substance P in offspring from WT mothers (n=6) but not in offspring from IL5tg mothers (n=6).

C6. Maternal IL-5 changes the mechanism of airway hyperreactivity

We next assessed whether increased airway innervation affected therapeutic responses. Serotonin can activate serotonin receptors on airway nerves to elicit reflex bronchoconstriction[179] or bind to serotonin receptors on airway smooth muscle[584] to directly cause bronchoconstriction. Airway hyperreactivity was completely blocked by the muscarinic antagonist atropine (Figure 12a), demonstrating that serotonin-induced bronchoconstriction was mediated by a vagal reflex and release of acetylcholine, as opposed to stimulating serotonin receptors on smooth muscle directly. Bronchoconstriction induced by inhaled methacholine (in animals with cut vagal nerves to eliminate reflexes), was not different between offspring of mothers with different genotypes, demonstrating that airway hyperreactivity in offspring from IL5tg mothers was not due to enhanced smooth muscle contractility (Figure 12b). We tested nerve reflexes by surgically cutting the vagus nerves and sensory tachykinins by pharmacologically blocking substance P receptors with a neurokinin-1 (NK1) antagonist CP99994. Vagotomy markedly suppressed airway hyperreactivity in WT offspring from IL5tg mothers (Figure 12d), but did not suppress airway hyperreactivity in HDM exposed WT offspring born to WT mothers (Figure 12c). Substance P causes bronchoconstriction both directly, by binding to NK-1 receptors on airway smooth muscle, and indirectly by potentiating parasympathetic nerve induced contraction[290]. Unlike vagotomy, the NK-1 antagonist markedly worsened airway hyperreactivity in WT offspring from WT mothers (Figure 12e), producing bronchoconstriction so severe that only two of six survived the full serotonin-dose

response curve. Similar to cutting vagus nerves, the NK-1 antagonist suppressed HDM-induced airway hyperreactivity in WT offspring born to IL5tg mothers (Figure 12f). These data demonstrate that maternal IL-5 increases offspring vagal reflexes that mediate airway hyperreactivity and dramatically change the efficacy of nerve-targeted therapies.

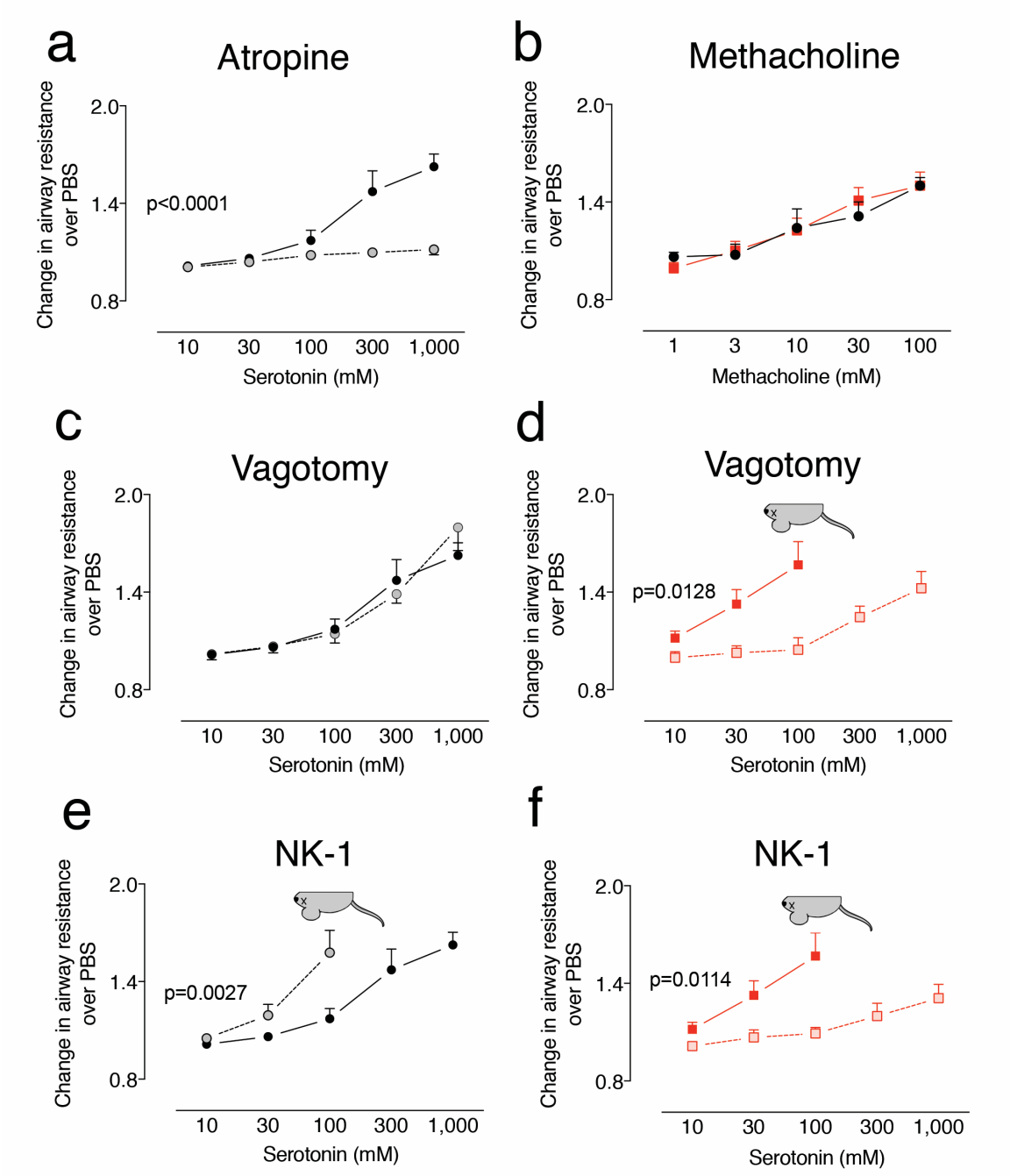
a
Atropine
Change in airway resistance over PBS
2.0
1.4
0.8
p<0.0001
10
30
100
300
1,000
Serotonin (mM)
b
Methacholine
Change in airway resistance over PBS
2.0
1.4
0.8
1
3
10
30
100
Methacholine (mM)
c
Vagotomy
Change in airway resistance over PBS
2.0
1.4
0.8
10
30
100
300
1,000
Serotonin (mM)
d
Vagotomy
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0128
10
30
100
300
1,000
Serotonin (mM)
e
NK-1
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0027
10
30
100
300
1,000
Serotonin (mM)
f
NK-1
Change in airway resistance over PBS
2.0
1.4
0.8
p=0.0114
10
30
100
300
1,000
Serotonin (mM)

Figure 12. Nerve-mediated airway hyperreactivity in offspring exposed to maternal IL-5. Serotonin-induced bronchoconstriction in HDM treated offspring (a, ●, n=8) was blocked by atropine (●, n=5, p<0.0001, F=8.87, df=4). Methacholine induced bronchoconstriction was not different between vagotomized HDM exposed offspring from WT (b, ●, n=7) and IL5tg (■, n=6) mothers. Cutting the vagus nerves (c, ●, n=8) did not inhibit serotonin-induced bronchoconstriction in HDM exposed WT offspring from WT mothers. Cutting the vagus nerves (d, □, n=6) suppressed hyperreactivity in HDM exposed WT offspring from IL5tg mothers (■=HDM exposed offspring from IL5tg mother intact vagus nerves, n=8, p=0.0128, F=5.259, df=2, inverted mouse denotes where death occurred). Blocking neurokinin-1 receptors (e, ●, n=6, p=0.0027, F=7.634, df=2) potentiated hyperreactivity in HDM exposed WT offspring from WT mothers. Blocking neurokinin-1 receptors (f, □, n=7, p=0.0114, F=5.334, df=2) suppressed hyperreactivity in HDM exposed WT offspring from IL5tg mothers. Data presented as mean ± SEM. Physiology data P values represent interaction term of repeated measure two-way ANOVA. Statistics for figure d-f calculated only for 10-100 mM doses.

C7. Offspring eosinophils are required for airway hyperreactivity.

Eosinophils are actively recruited to nerves via neuronal eotaxin[372] and increase sensory nerve branching[207]. However, nodose-jugular neurons also express IL-5 receptors and respond to exogenous IL-5[585]. To test whether nerve growth and airway responsiveness required fetal eosinophils, we crossed IL5tg female mice with eosinophil-deficient male mice (Figure 8a)[35], producing offspring that had been exposed to maternal IL-5 *in utero* but had no eosinophils. Eosinophil deficiency prevented HDM-induced airway hyperreactivity in all offspring regardless of maternal genotype (Figure 13a). Eosinophil-deficiency also prevented increased nerve length and growth due to maternal IL-5 (Figure 13b). To test whether maternal IL-5 caused fetal eosinophilia, we collected fetal blood and amniotic fluid from IL5tg and WT mothers between gestation days 18-20. In WT mother gestations, amniotic fluid IL-5 was undetectable and there were no blood eosinophils in these fetuses (Figure 13c-e). In contrast, WT fetuses from IL5tg mothers had elevated IL-5 in amniotic fluid and peripheral blood eosinophilia (Figure 13c-e). These data demonstrate that maternal IL-5 does not directly cause airway hyperreactivity in offspring and instead requires eosinophil hematopoiesis and recruitment to the airway.

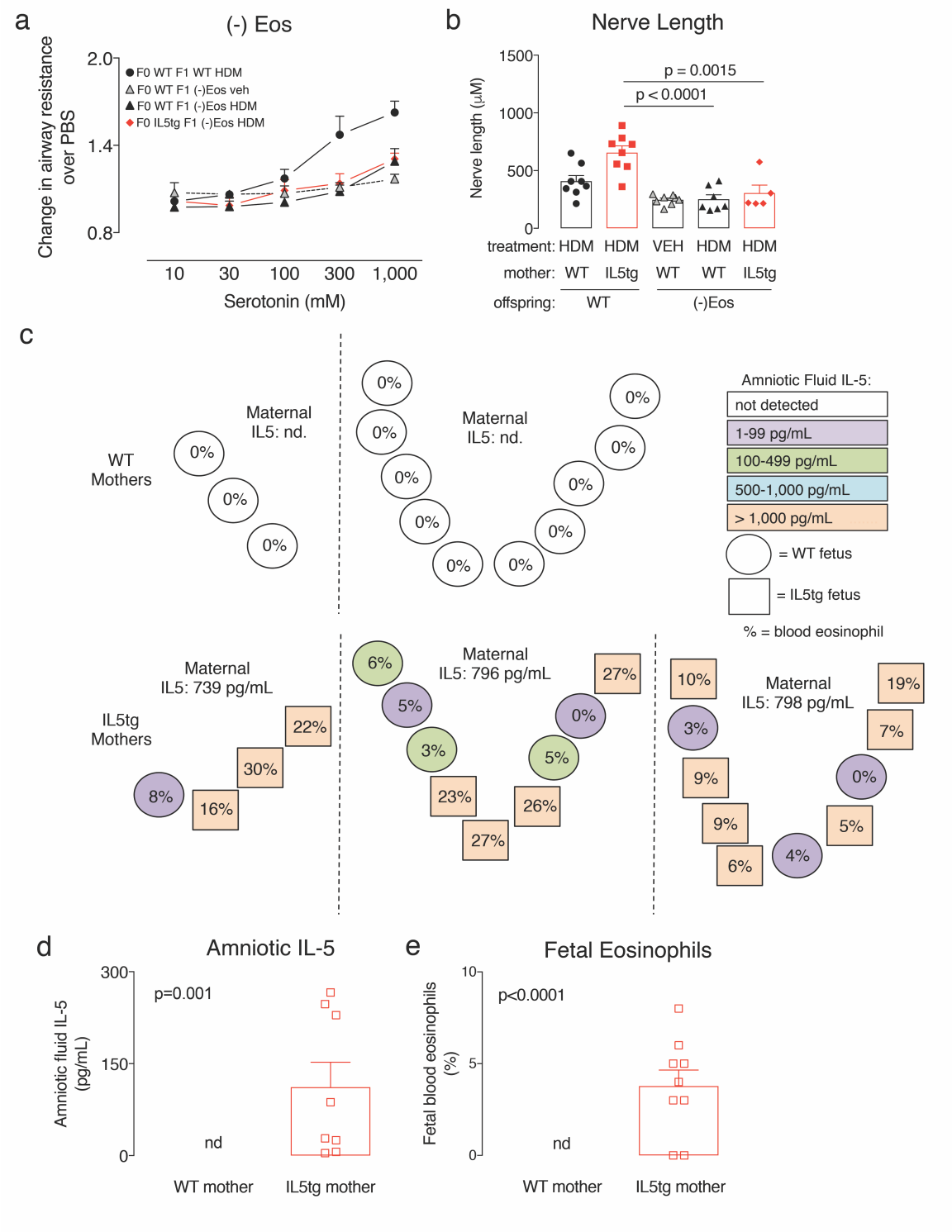
a
(-) Eos
Change in airway resistance over PBS
2.0
1.4
0.8
F0 WT F1 WT HDM
F0 WT F1 (-)Eos veh
F0 WT F1 (-)Eos HDM
F0 IL5tg F1 (-)Eos HDM
10
30
100
300
1,000
Serotonin (mM)
b
Nerve Length
Nerve length (μM)
1500
1000
500
0
p = 0.0015
p < 0.0001
treatment: HDM HDM VEH HDM HDM
mother: WT IL5tg WT WT IL5tg
offspring: WT (-)Eos
c
WT Mothers
Maternal IL5: nd.
0%
0%
0%
Maternal IL5: nd.
0%
0%
0%
0%
0%
0%
0%
0%
Amniotic Fluid IL-5:
not detected
1-99 pg/mL
100-499 pg/mL
500-1,000 pg/mL
> 1,000 pg/mL
= WT fetus
= IL5tg fetus
% = blood eosinophil
IL5tg Mothers
Maternal IL5: 739 pg/mL
8%
16%
30%
22%
Maternal IL5: 796 pg/mL
6%
5%
3%
23%
27%
26%
5%
0%
27%
Maternal IL5: 798 pg/mL
10%
3%
9%
9%
6%
4%
5%
0%
7%
19%
d
Amniotic IL-5
Amniotic fluid IL-5 (pg/mL)
300
150
0
p=0.001
nd
WT mother
IL5tg mother
e
Fetal Eosinophils
Fetal blood eosinophils (%)
10
5
0
p<0.0001
nd
WT mother
IL5tg mother

Figure 13. Eosinophils were required for offspring airway hyperreactivity and hyperinnervation. Congenital eosinophil deficiency [(-)Eos] suppressed airway hyperreactivity in HDM exposed offspring from both WT (a, ▲, n=7) and IL5tg mothers (◆, n=5; vehicle: ▲, n=8) (WT HDM vs (-)Eos HDM, p=0.0101, t=3.536, df=24). Eosinophil deficiency suppressed airway sensory hyperinnervation in offspring exposed to maternal IL-5 (b). Amniotic fluid IL-5 and peripheral eosinophils were elevated in WT fetuses of IL5tg mothers (c). Each cluster of circles and squares represents one pregnant mouse, so n=2 WT mothers and n=3 IL5tg mothers. Each circle or square represents one fetus, with circles denoted WT fetuses and squares denoting IL5tg fetuses, while spatial orientation denotes fetal position *in utero*. Circle or square shading represents amniotic IL-5 level and percentage represented fetal blood smear eosinophil count. Maternal serum IL-5 denoted above each cluster of fetuses. Graphical representation of amniotic fluid IL-5 (d; WT offspring from WT mother, n=13; WT offspring from IL5tg mother n=9) and fetal eosinophil count (e). Data presented as mean ± SEM. Nerve length P value represents Sidak post-test of one-way ANOVA of HDM treated offspring. nd= not detected.

D. Discussion

Sensory neurons are required for airway hyperreactivity[186] and my data demonstrate that maternal and fetal inflammatory responses increase lung innervation, enhancing baseline airway responses. Offspring from IL5tg mothers had the greatest innervation, with baseline airway hyperreactivity and the most severe hyperreactivity after allergen exposure that was independent of changes in inflammatory cytokines. A certain amount of increased innervation is clearly required for heightened responses at baseline, as offspring from HDM exposed mothers were not hyperreactive at baseline and innervation levels in these animals were comparable to WT offspring from WT mothers (Figure 14). Thus I postulate that increased lung innervation in offspring of mothers exposed to HDM or with elevated IL-5 sets the stage for postnatal allergen sensitization and inflammatory cell recruitment to cause airway hyperreactivity. This is important because heightened airway responses in early infancy predict later development of asthma in humans[94].

My data show that maternal HDM exposure or IL-5 changes the physiological mechanism of airway hyperreactivity in adult offspring, driven by structural and functional changes in airway sensory nerves and long lasting increases in neurotrophins. While elevated BDNF, which persists into adulthood in offspring of HDM exposed mothers, is only associated with airway hyperinnervation, it may be significant since BDNF correlates with asthma severity in humans[471,472] and the BDNF promoter is differentially methylated in children with asthma compared

to children without asthma[103]. Increased NGF in offspring from IL5tg mothers is also likely functionally significant since overexpression of NGF in airway epithelium increases sensory innervation and causes airway hyperreactivity in mice[6]. With regards to other neurotrophins, I observed no change in NT4 or GDNF levels. However, NT4 increases airway innervation after allergen exposure in the immediate postnatal period[535,582], suggesting the timing of inflammatory insults activates distinct neurotrophin pathways. Furthermore, GDNF promotes parasympathetic ganglia migration and development *ex vivo* in mice[503]. Since neither parasympathetic ganglia structure nor lavage GDNF were affected by maternal HDM exposure or IL-5, my results suggest a targeted effect of maternal inflammation on sensory nerve development of the offspring.

Despite promising pre-clinical trials of neurokinin receptor antagonists in animal models of asthma, blocking neurokinin receptors in patients with asthma worsened allergen-induced airway responses[307]. My data suggest a novel, protective role for substance P in airway hyperreactivity that is lost with IL-5 exposure *in utero*, which may contribute to the failure of NK1 antagonists clinically. How IL-5 changes the role of substance P in airway hyperreactivity is not clear. Offspring from WT mothers had less substance P compared with offspring from IL5tg mothers, so it is possible that below a certain expression level substance P-receptor signaling causes bronchodilation. Activation of afferent sensory nerves can elicit peripheral nerve reflexes to cause NK1-dependent, NANC relaxation of airway smooth muscle[165]. Substance P also

relaxes pre-contracted airway smooth muscle via epithelial release of prostaglandins[586,587]. However, blocking epithelial NK1 receptors with inhaled CP99994 had no effect on serotonin-induced bronchoconstriction (Figure 15), suggesting that *in utero* exposure to IL-5 affects nerve or smooth muscle substance P signaling.

Active allergen exposure during pregnancy was not required for offspring airway hyperreactivity since mothers exposed to HDM gave birth to a second litter and founded a second generation of offspring that were also hyperreactive. Airway inflammation after allergen exposure resolves within 4 weeks in mice[7,583]. Mothers were exposed to HDM for 4 weeks before and then for the duration of their first pregnancy. HDM exposure stopped once the first litter was born and the mothers immediately became pregnant with a second litter. Thus, offspring from a second pregnancy are developing during this 4-week window of inflammation resolution. The persistence of airway hyperreactivity in offspring from a second pregnancy therefore may simply reflect on-going maternal inflammation.

Conversely, inflammation can permanently reset homeostatic processes[588]. The field of metaflammation[589] best explains this concept, albeit in a different disease context. Macrophages infiltrate into adipose tissue during obese states and secrete TNFα[590]. High levels of TNFα cause insulin resistance[591], resetting the homeostatic set point of blood glucose. Abstracting this concept to asthma, it is possible that the maternal inflammatory response to HDM reset a homeostatic

process important for fetal development. Eosinophils are present in uterine tissue[592,593], thus it is conceivable that eosinophil interactions with uterine stromal cells may have changed in response to maternal inflammation, thus subsequently affecting fetal development during the second pregnancy.

The persistence of airway hyperreactivity to a second pregnancy and second generation could also result from epigenetic modifications of nascent oocytes. Differential methylation has been found in newborns who develop asthma during adolescence compared with newborns who never develop asthma[102,103], suggesting modulation of gene expression, as opposed to inheritance of specific risk alleles, can increase asthma risk. For example, hypomethylation of IL-13 has been found in children with asthma[108] [insert Yang citation], which would presumably increase IL-13 production and induce eosinophilic lung inflammation, IgE synthesis, airway hyperreactivity, and mucus production[22]. However, it has not directly been tested whether methylation of a specific gene causes pathologic features of asthma. My study provides a suitable experimental platform to test whether DNA is differentially methylated in offspring descended from mothers exposed to HDM and whether reversing DNA methylation at specific genomic sites can prevent airway hyperreactivity. These studies are ongoing.

While not powered to detect sex differences, I observed that male mice in all groups had greater hyperreactivity at baseline and were more severely affected by maternal HDM exposure than female mice. Males are more likely than

females to be diagnosed during childhood with asthma[69], suggesting that increased baseline airway reactivity may cause more apparent clinical disease and lead to an earlier diagnosis. Male and female fetuses and their placenta also develop differently, with males showing less adaptation to maternal stressors and increased sensitivity to environmental insults during pregnancy than females[594]. Maternal asthma changes the placental transcriptome[595] in a sex-specific manner and amniotic fluid collected from male gestations has more IL-5 than female gestations[596]. Thus, placental maladaptation and increased inflammatory cytokines may explain why maternal allergen exposure more severely affects male airway development compared with females.

Nerves secrete chemoattractant factors[372] that attract immune cells into close association[373,597,598]. Nerves also secrete neurotransmitters that directly activate immune cells and potentiate inflammatory responses. For example, substance P causes eosinophil degranulation and release of major basic protein[280] that inhibits M2 muscarinic receptors, while other neuropeptides including neuromedin U enhance type 2 innate lymphoid cell inflammatory responses[599]. Thus increased airway innervation in offspring from HDM exposed or IL5tg mothers may have increased offspring airway inflammation, which established a positive feed forward loop to increase airway hyperreactivity.

The role of eosinophils in airway hyperreactivity remains controversial since eosinophil-targeted therapies, while effective at reducing exacerbations[54], do not

reduce airway hyperreactivity in adults with asthma[600]. Eosinophils were required for nerve changes in offspring exposed to maternal IL-5, uncovering a novel role for eosinophils in shaping airway neurodevelopment. My data in adult offspring suggest that airway hyperreactivity is mediated by nerve development and thus not responsive to eosinophil-targeted therapies later in life.

In summary, I propose a novel mechanism of *in utero* programming of airway hyperreactivity that is responsive to maternal HDM exposure and IL-5 and dependent on structural changes to airway nerves that persist into adulthood (Figure 16). My data suggest that excessive maternal type-2 inflammation is an important *exposure* factor for the development of asthma, which may be amenable to intervention. Better management of asthma in pregnant women may decrease the likelihood of airway hyperreactivity in their children.

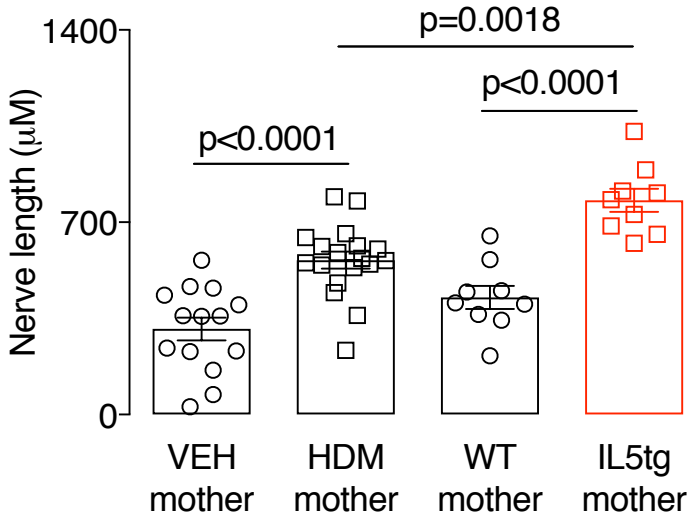

Figure 14. Maternal IL-5 increased offspring airway innervation more than maternal HDM exposure. Epithelial nerve length increased in offspring from HDM exposed mothers compared with vehicle-exposed mothers, but not as much as offspring exposed to maternal IL-5. Offspring from VEH mother, n=14; Offspring from HDM mother, n=18; Offspring from WT mother, n=9; Offspring from IL5tg mother, n=9).

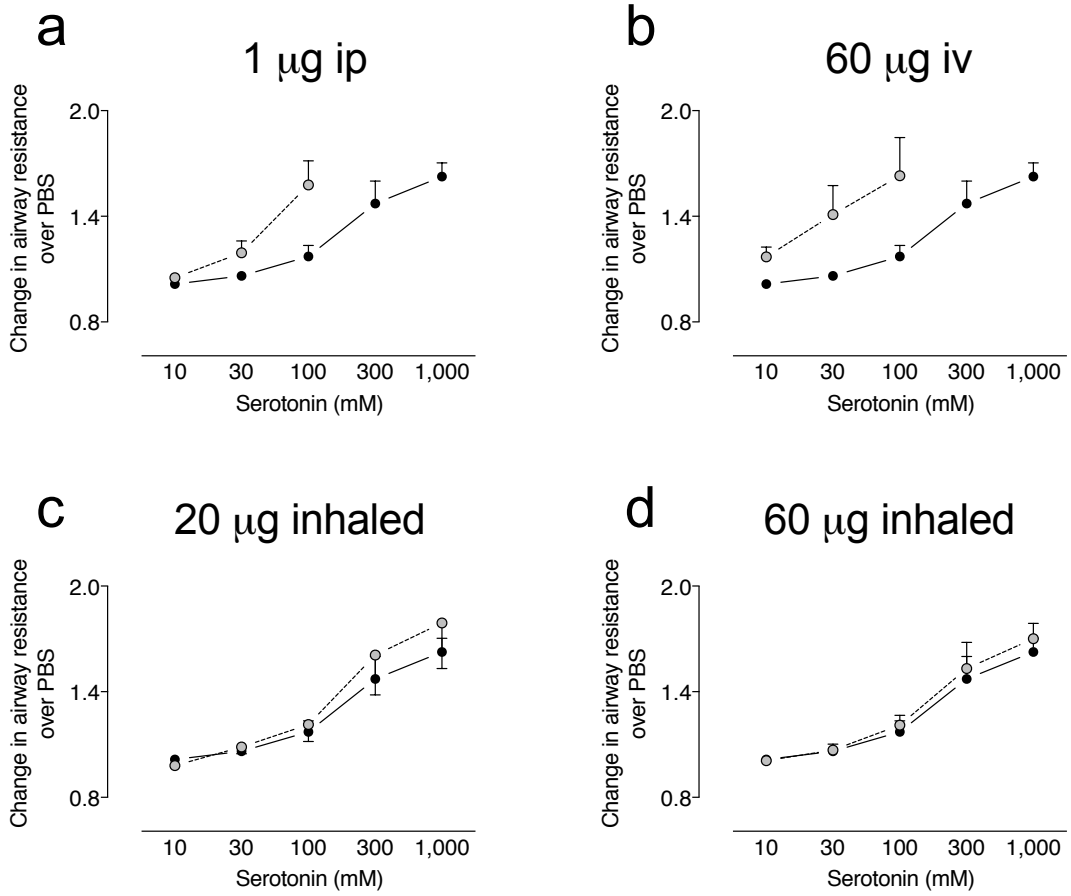

Figure 15. Blocking epithelial neurokinin-1 receptors did not affect airway hyperreactivity. The competitive NK1 antagonist, CP99994, was delivered systemically (a-b; grey circles) or via a nebulizer (c-d; grey circles) 15 minutes before a serotonin-dose response curve. One (n=6) and 60 μg (n=4) of systemic CP99994 potentiated airway hyperreactivity in HDM-exposed mice. Twenty (n=5) or 60 μg (n=7) of inhaled CP99994 had no affect on airway hyperreactivity in HDM-exposed mice.

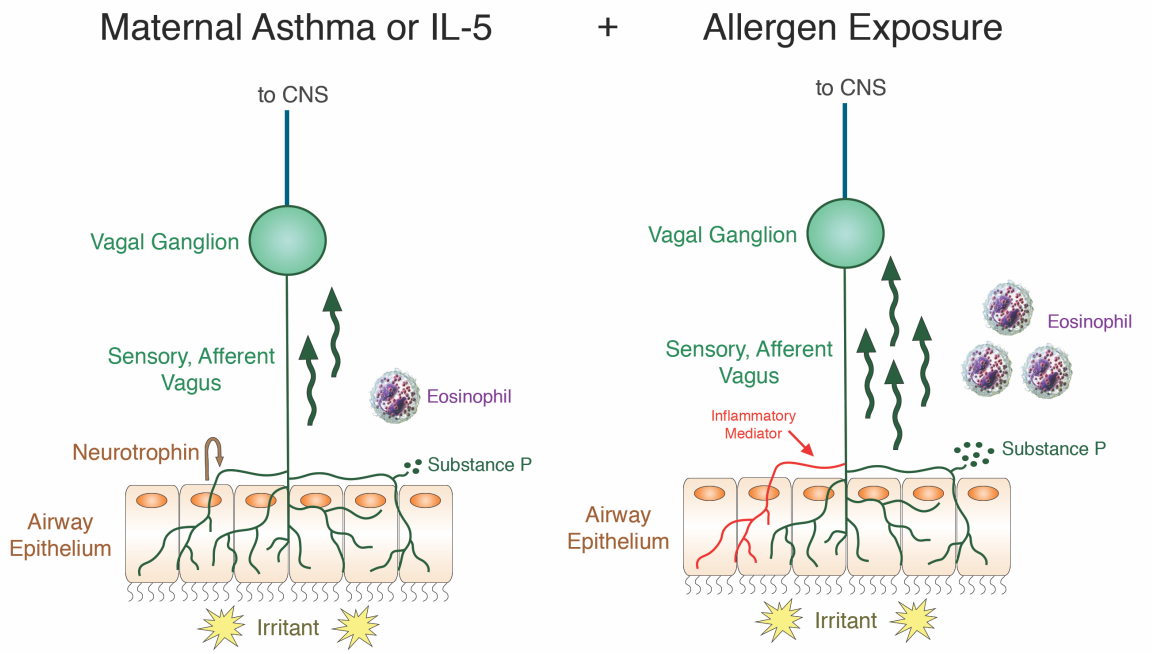

Figure 16. Schematic of chapter 3 results. Reflex bronchoconstriction is increased in offspring born to mothers exposed to HDM or with elevated IL-5. Heightened reflex bronchoconstriction is likely due to increased airway sensory innervation and release of substance P observed in offspring born to mothers exposed to HDM or IL-5. Epithelial neurotrophin expression also increased in offspring born to mothers exposed to HDM or IL-5, which may sustain increased structural complexity and activity of airway nerves. Eosinophils are required for offspring airway hyperreactivity, but whether eosinophils interact directly or indirectly with nerves, and what eosinophils secrete to change nerve development, are unknown. When offspring are exposed to allergen themselves later in life, increased lung innervation sets the stage for more severe airway hyperreactivity, increased neurotransmitter release, and augmented immune cell recruitment. Eosinophils adapted from Wikipedia.org.

E. Methods

E1. Mating strategy

N=10 nulliparous female C57Bl/6J mice were randomized to receive HDM (n=5) or vehicle (n=5). N=5 mice were chosen since C57Bl/6 mice have 7 offspring per litter on average and n=24 offspring were required for experiments. Maternal allergen exposure protocol is detailed in general methods section (C. Allergen Sensitization and Exposure). Once the first litter of offspring was born, maternal exposure was stopped. Female and male mice were housed together while the first litter (F1 challenge) nursed. During this time, the female became pregnant again and subsequently gave birth to a second litter (F1 post-challenge).

To produce the second generation of mice, F1 female (n=2) and F1 males (n=2) from different HDM exposed mothers and F1 female (n=2) and F1 males (n=2) from different vehicle exposed mothers were bred starting at 8 weeks of age. F2 offspring from the first three litters were used for experiments.

E2. Maternal physiology

A second cohort of female mice (n=30) was randomized to receive HDM (n=15) or vehicle (n=15) and exposed as described in general methods section. In this cohort, one HDM exposed and one vehicle exposed mouse did not become pregnant and were used for physiology experiments (Figure 3). In addition, two HDM exposed and two vehicle exposed mothers euthanized their pups 2 days after birth, thus these mice were also used for maternal physiology experiments.

E3. Amniotic fluid collection

Amniotic fluid was collected from pregnant, HDM or vehicle exposed mothers from the second (n=30) cohort of mice as described in the general methods section.

E4. Statistical analysis

Data graphed as mean ± SEM and analyzed using GraphPad Prism 7. All groups contained n=5-10 animals with specifics denoted in figure legends. Airway resistance data were analyzed with a repeated-measures two-way ANOVA. Reported *p* values represent interaction term of two-way ANOVA (genotype or treatment x serotonin dose response). For experiments where mice died during the serotonin-dose response curve, data graphed and analyzed only where all animals were still living (denoted in graphs). For HDM-exposed WT offspring from IL5tg mothers, n=3 died prior to 300 mM serotonin, n=4 died prior to 1000 mM dose, and n=1 survived the dose response curve. For HDM-exposed, NK-1 treated WT offspring from WT mothers, n=2 died prior to 300mM serotonin, n=2 died prior to 1000mM dose, and n=2 survived the dose response curve. All other data were analyzed with a two-way ANOVA with Sidak's posttest and reported *p* values represent post-test statistical value.

CHAPTER 4. Postnatal eosinophilic inflammation arrests airway parasympathetic nerve development.

A. Abstract

Humans are born with immature lungs with substantial structural development continuing after birth. Immunologic adaptation to a new, inhaled environment occurs in parallel with structural development and inflammatory insults during this critical window influence risk for respiratory diseases[601], including asthma[564]. Innate immune cells normally influx into the lungs after birth[538-540] and have the potential to influence lung development. Here I show that airway parasympathetic cholinergic ganglia continue to develop after birth and contain rare populations of neurons that express neurotransmitters not normally expected in these ganglia, including substance P, neuronal nitric oxide synthase (which produces nitric oxide), and tyrosine hydroxylase (which produces catecholamines). Increasing eosinophil influx into the lungs after birth arrests parasympathetic ganglia development, resulting in fewer ganglia, less heterogeneous neurotransmitter expression, and reduced airway hyperreactivity. Deletion of eosinophils prevents airway hyperreactivity, with maximal airway parasympathetic ganglia formation and neurotransmitter heterogeneity. These data demonstrate that airway parasympathetic ganglia are susceptible to eosinophilic inflammation in the postnatal environment that subsequently influence development of airway hyperreactivity.

B. Introduction

Airway nerves mediate bronchoconstriction. Efferent vagal nerves synapse on a complex network of parasympathetic ganglia embedded within the airway, activating ganglia to release acetylcholine, which contracts airway smooth muscle. Blocking parasympathetic nerve activity with muscarinic antagonists including tiotropium improves lung function in adults[182] and children with severe asthma[578], demonstrating the importance of airway innervation for optimal lung health.

Airway innervation continues to develop after birth, with increases in sensory innervation[524-528] and sensitivity to contractile agonists[529-531], and decreases in relaxant innervation[530,532]. Innervation comparable to adults is reached within a few weeks after birth for mice. Environmental insults during this critical period permanently alter airway innervation and promote airway hyperreactivity later in life. For example, postnatal ovalbumin[535] or cigarette smoke[536] exposure increases airway innervation with subsequent airway hyperreactivity in mice. The postnatal period represents a particularly sensitive time period, since environmental exposures to allergen or pollution later in life do not alter lung innervation or cause persistent airway hyperreactivity as they do in neonates[526,529,536].

Neonatal airway immune responses to environmental insults are intrinsically skewed towards type 2 inflammation. The first breath of a neonate induces IL-33 secretion from airway epithelial cells that activates a cascade of airway type 2

innate lymphoid cell expansion, IL-5 and IL-13 secretion, and recruitment of peripheral eosinophils into the airway[538-540]. This postnatal influx of eosinophils occurs normally and cells decline to adult levels after a couple of weeks in mice. In adults, airway eosinophils interact with airway nerves and suppress M_2-muscarinic receptor function to increase acetylcholine release[373]. Here I tested whether parasympathetic cholinergic ganglia continue to develop after birth and are vulnerable to postnatal lung eosinophilia.

C. Results

C1. Eosinophils suppress airway parasympathetic ganglia in adults

I assessed parasympathetic cholinergic ganglia number in adult, WT mice and mice congenitally deficient in eosinophils [(-)Eos] or with airway hypereosinophilia (IL5tg) due to overexpression of IL-5. Whole-mount tracheas were stained with a pan-neuronal marker, PGP9.5, optically cleared and imaged from vocal cords to mainstem bronchi (Figure 17a). Maps of airway ganglia were created for each animal (Figure 17b). Parasympathetic ganglia decrease rapidly in distal airways, with none detected after second or third branch level. Thus, focusing on upper airway ganglia allowed for an easily defined anatomical space, while capturing the majority of ganglia.

Parasympathetic ganglia formed a large, complex network on the posterior surface of the trachea, with over 70 individual ganglia ranging in size from single neurons to dense, highly populated clusters (Figure 17c-f). Ganglia number, size,

and pattern varied considerably between WT mice, consistent with previous reports[602]. Mice deficient in eosinophils had significantly increased numbers of parasympathetic ganglia compared with WT mice (Figure 17g). IL5tg mice had significantly decreased numbers of parasympathetic ganglia compared with WT mice (Figure 17g), demonstrating a correlation between parasympathetic ganglia number and airway eosinophils.

To test if increased parasympathetic ganglia in (-)Eos mice represented a change of neuron clustering into different sized ganglia, number of neurons were counted and ganglia divided into groups with less than 5 neurons, 6-19 neurons, or more than 20 neurons (Figure 17h-j). Individual neurons were difficult to count accurately in ganglia with more than 20 cell bodies, thus all ganglia with more than 20 neurons were grouped together. (-)Eos mice had significantly more ganglia consisting of less than 5 neurons or 6-19 neurons, with no change in larger ganglia, compared with WT mice. IL5tg mice had significantly fewer ganglia consisting of less than 5 neurons, with no change in larger ganglia, compared with WT mice.

These data led me to hypothesize that eosinophils affect neuron clustering and suppress migration of neurons away from larger ganglia. However, an alternative explanation is that eosinophils suppress stem cell division, resulting in fewer neurons that could have formed smaller ganglia. To test this, I attempted to dissociate ganglia and count individual neurons *in vitro*, however, these

experiments were unsuccessful due to technical limitations. Thus, these hypotheses remain to be tested.

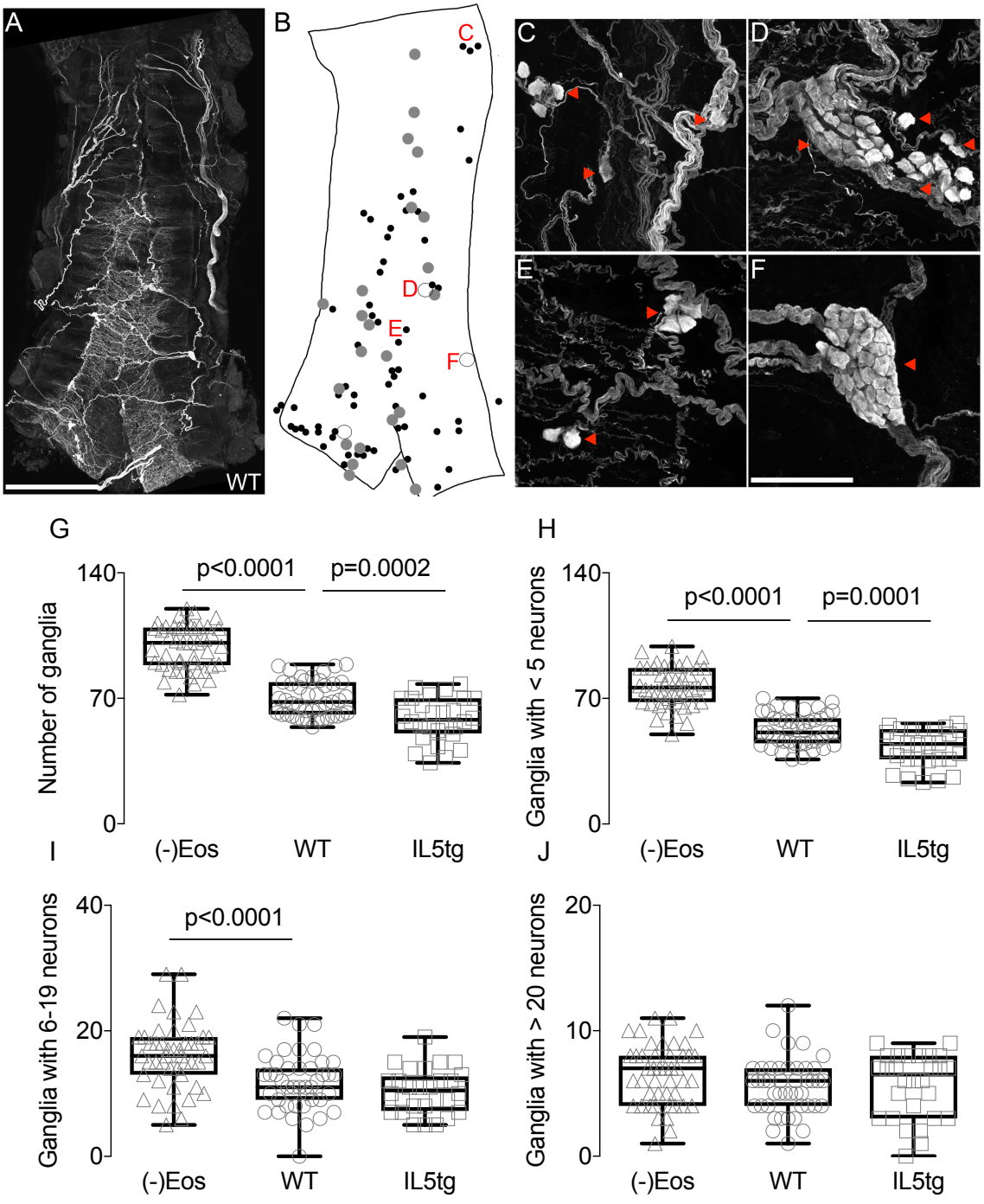
A
WT
B
C
D
E
F
C
D
E
F
G
Number of ganglia
140
70
0
p<0.0001
p=0.0002
(-)Eos
WT
IL5tg
H
Ganglia with < 5 neurons
140
70
0
p<0.0001
p=0.0001
(-)Eos
WT
IL5tg
I
Ganglia with 6-19 neurons
40
20
0
p<0.0001
(-)Eos
WT
IL5tg
J
Ganglia with > 20 neurons
20
10
0
(-)Eos
WT
IL5tg

Figure 17. Airway eosinophils decreased parasympathetic ganglia. **(A)** Maximum intensity projection of whole-mount, optically cleared wild-type mouse trachea stained with pan-neuronal marker PGP9.5. Trachea is oriented proximal at top and mainstem bronchi at bottom. Scale bar = 1500μm. **(B)** Map of parasympathetic ganglia from trachea in A. Black circles represent ganglia with 1-5 neurons. Grey circles represent ganglia with 6-19 neurons. Clear circles represent ganglia with 20+ neurons. Letters indicate locations of images C-F. Examples of a small ganglion and solitary neurons **(C)**, medium and small ganglia **(D)**, small ganglia **(E)**, and a large ganglion **(F)** scale bar = 100 μm. Red triangles point to ganglia (**C-F**). Eosinophils decreased total parasympathetic ganglia **(G)**, affecting predominantly small ganglia **(H)** and not medium **(H)** or large **(J)** ganglia. (-)Eos n= 45, WT n=43, IL5tg n=28. Data graphed as box plots, with ends of box representing upper and lower quartile, median denoted as solid middle line, and whiskers identifying maximum and minimum data points.

C2. Rare neurons within parasympathetic ganglia express non-classical neurotransmitters that are suppressed by eosinophils

Expression of choline acetyltransferase and synthesis of acetylcholine defines parasympathetic cholinergic nerves. However, neurotransmitter heterogeneity within parasympathetic cholinergic ganglia in other organs has been reported, with individual neurons expressing substance P[603], tyrosine hydroxylase[604-606] and nNOS[607]. Only substance P[219,225-227,603] has consistently been reported in tracheal parasympathetic ganglia. I confirmed that rare neurons within tracheal parasympathetic ganglia expressed substance P (Figure 18a) and also identified additional neurons that expressed nNOS (Figure 18b) and tyrosine hydroxylase (Figure 18c). Co-localization of substance P with choline acetyltransferase confirmed that neurons expressed dual cholinergic and sensory phenotypes (Figure 19).

To test if eosinophils changed the prevalence of these rare neurons, PGP9.5 positive ganglia (Figure 17g) were co-stained with substance P, nNOS, or tyrosine hydroxylase. Eosinophil deficient mice had more neurons expressing substance P (Fig 18d), nNOS (Figure 18e), and tyrosine hydroxylase (Figure 18f) than WT mice, however elevated eosinophils in IL5tg mice did not further suppress neurotransmitter heterogeneity.

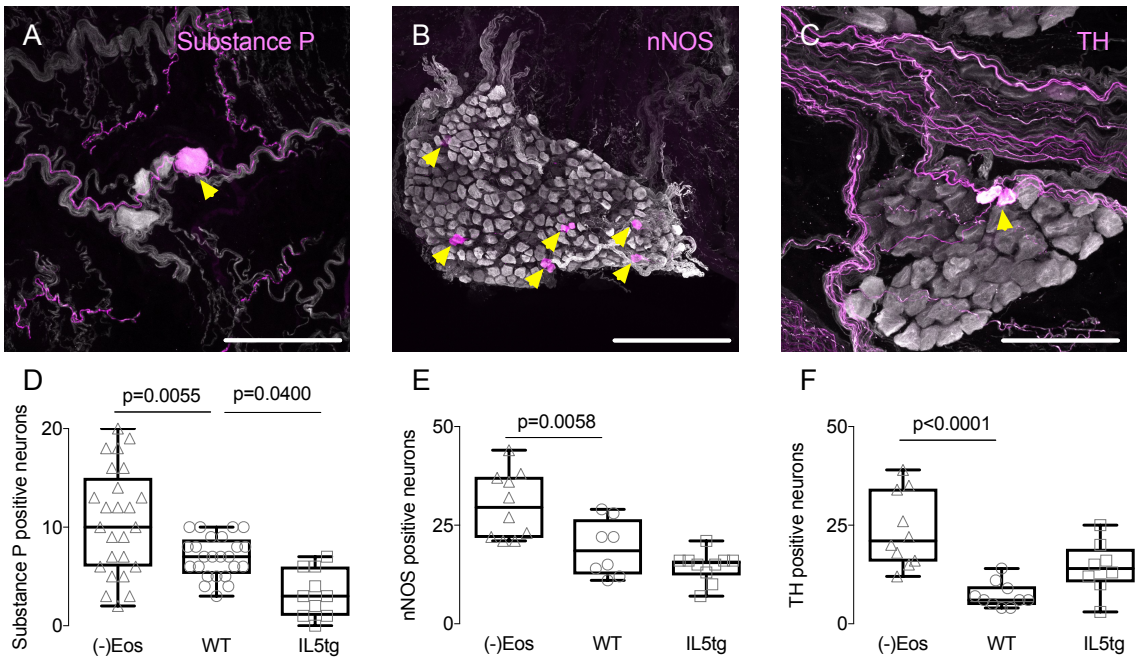

Figure 18. Eosinophils suppressed neurotransmitter heterogeneity of parasympathetic ganglia neurons. Rare neurons (identified with yellow arrowhead) within parasympathetic ganglia express substance P (**A,** Scale bar = 75 μm), nNOS (**B,** Scale bar = 150 μm), or tyrosine hydroxylase (TH, **C,** Scale bar = 75 μm). Eosinophils suppress the expression of substance P (**D**, (-)Eos n=25, WT n=24, IL5tg n=10), nNOS (**E**, (-)Eos n=10, WT n=8, IL5tg n=10), and tyrosine hydroxylase (**F**, (-)Eos n=10, WT n=11, IL5tg n=8) by parasympathetic ganglia neurons. Data show number of neurons/trachea, graphed as box plots, with ends of box representing upper and lower quartile, median denoted as solid middle line, and whiskers identifying maximum and minimum data points.

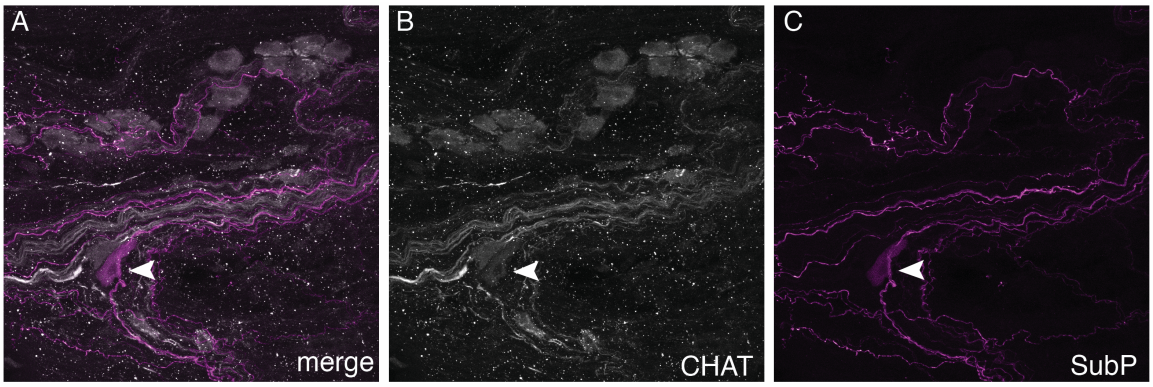

Figure 19. Substance P and choline acetyltransferase (CHAT) co-localized in airway ganglia. (a) a neuron within airway parasympathetic ganglia (arrow head) stained positive for both CHAT (white) and substance P (magenta). (b) CHAT staining alone. (c) Substance P staining alone. Image is 63x magnification, 220x220 μm, whole-mount airway parasympathetic ganglia from WT mouse.

C3. Eosinophils suppress parasympathetic ganglion expansion after birth

Pelvic parasympathetic ganglia expand after birth, with a doubling in the number of ganglia between birth and postnatal day 21[608]. Thus, I tested whether differences in airway parasympathetic ganglia in adult mice with and without eosinophils occurred after birth. Airways were collected on postnatal day 1, 7, and 21 and parasympathetic ganglia counted. Parasympathetic ganglia number were the same between (-)Eos, WT, and IL5tg mice on postnatal day 1 (Figure 20a), demonstrating that eosinophil suppression of parasympathetic ganglia occurs after birth. A near doubling in ganglia number occurred in (-)Eos mice between postnatal day 1 and adulthood (Figure 20b). WT mice also exhibited a rapid increase in parasympathetic ganglia between postnatal day 1 and adulthood (Figure 20c). In contrast, parasympathetic ganglia did not change in IL5tg mice between postnatal day 1 and adulthood (Figure 20d), suggesting eosinophils prematurely arrest parasympathetic ganglia development.

Neurotransmitter heterogeneity also changed after birth. Substance P expressing neurons did not change between postnatal day 1 and adulthood (Fig 21a), while nNOS expressing neurons continued to expand (Figure 21b) and tyrosine hydroxylase expressing neurons decreased (Figure 21c). Experiments testing whether suppression of neurotransmitter heterogeneity by eosinophils occurs after birth are ongoing.

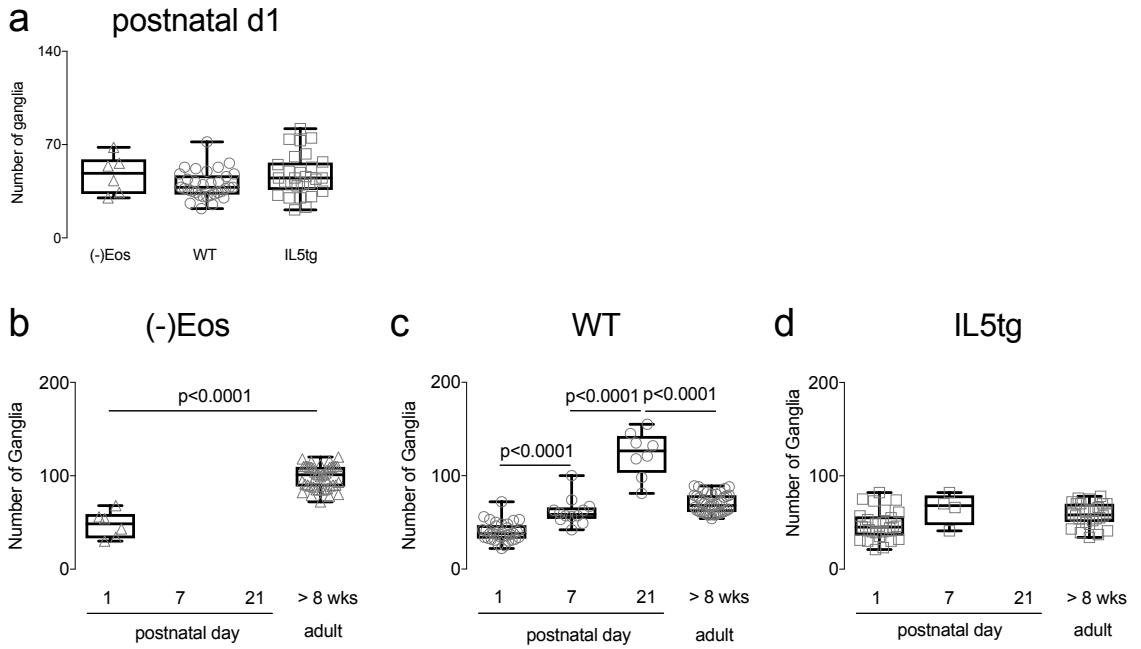

Figure 20. Eosinophils suppressed parasympathetic ganglia expansion after birth. (a) Parasympathetic ganglia were the same between (-)Eos, WT, and IL5tg mice on postnatal day 1. (b) Parasympathetic ganglia increased between postnatal day 1 and adulthood in (-)Eos mice (postnatal day 1 n=6; adulthood n=45). (c) Parasympathetic ganglia increased between postnatal day 1 and adulthood in WT mice (postnatal day 1 n=33; postnatal day 7 n=12; postnatal day 21 n=8; adulthood n=43). (d) Parasympathetic ganglia did not increase between postnatal day 1 and adulthood in IL5tg mice (postnatal day 1 n=30; postnatal day 7 n=4; adulthood n=28). Data graphed as box plots, with ends of box representing upper and lower quartile, median denoted as solid middle line, and whiskers identifying maximum and minimum data points.

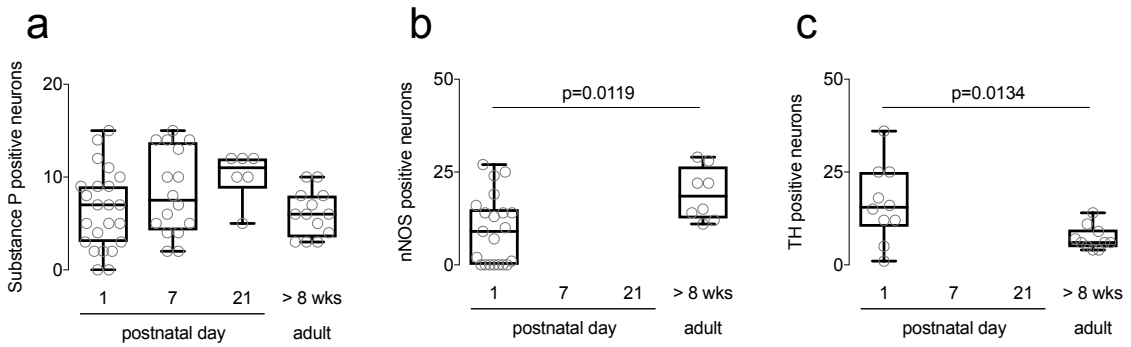

Figure 21. Parasympathetic neuron heterogeneity changed after birth. (a) Substance P expressing parasympathetic neurons did not increase after birth in WT mice (postnatal day 1 n=23; postnatal day 7, n=17; postnatal day 21, n=6; adult, n=24). (b) nNOS expressing parasympathetic neurons increased after birth (postnatal day 1, n=21; adulthood, n=8). Tyrosine hydroxylase expressing neurons decreased after birth (postnatal day 1, n=10; adulthood, n=10). Data graphed as box plots, with ends of box representing upper and lower quartile, median denoted as solid middle line, and whiskers identifying maximum and minimum data points.

C4. Airway eosinophils decrease airway smooth muscle contractility

Muscarinic receptors continue to develop after birth with enhanced contractility to methacholine in the neonatal period compared to adulthood[529]. Eosinophils increase reflex bronchoconstriction[206], which may result from increased sensory innervation and afferent activation (Chapter 3), increased efferent signals due to deranged parasympathetic ganglia formation described above, M_2-muscarinic receptor inhibition by major basic protein in the absence of allergen exposure[43,158], or enhanced smooth muscle sensitivity to acetylcholine. To test if eosinophils increased smooth muscle contractility, adult (-)Eos, WT and IL5tg mice were mechanically ventilated, vagal nerves cut to eliminate nerve reflexes, and exposed to increasing concentrations of inhaled methacholine to contract smooth muscle. Methacholine-induced bronchoconstriction was the same in (-)Eos and WT mice (Figure 22). However, IL5tg mice had reduced bronchoconstriction after inhaled methacholine. These data suggest that eosinophils increase reflex bronchoconstriction by increasing afferent nerve activation and suppressing parasympathetic ganglia formation, instead of enhancing smooth muscle contractility.

C5. M_2-muscarinic receptor inhibition does not potentiate serotonin-induced bronchoconstriction

Eosinophils suppress outgrowth of differentiating cholinergic neurons *in vitro*[609], an effect mimicked by exposing cholinergic neurons to purified major basic protein. Knockout of major basic protein in mice does not prevent allergen-

induced airway hyperreactivity, but it does decrease methacholine induced bronchoconstriction in the absence of allergen exposure[43]. Thus, I hypothesized that inhibition of M_2-muscarinic receptors by major basic protein during development may mediate suppression of parasympathetic ganglia expansion. To test this hypothesis, I could use major basic protein knockout mice or inhibit M_2-muscarinic receptors in WT mice pharmacologically with gallamine, an M_2-muscarinic receptor antagonist, and test changes in parasympathetic ganglia formation. I decided to test my hypothesis using a pharmacologic approach, but first tested whether gallamine potentiated airway responses to serotonin in untreated adult mice as a positive control for M_2-muscaranic receptor activity and correct gallamine dosing. I administered gallamine (1 mg/kg iv) to WT mice and measured bronchoconstriction after increasing doses of serotonin. Gallamine did not increase serotonin-induced bronchoconstriction (Figure 23). Major basic protein knockout also has no effect on serotonin induced bronchoconstriction[43], thus a different positive control is needed or major basic protein knockout mice are required to test if inhibition of M_2-muscarinic receptors suppresses postnatal parasympathetic ganglia development.

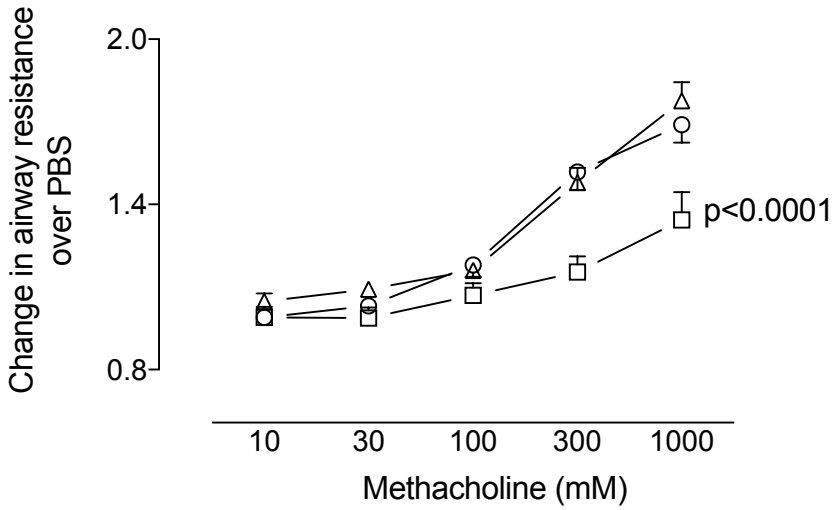

Figure 22. Airway eosinophils decreased methacholine-induced smooth muscle contraction. (-)Eos, WT and IL5tg mice were mechanically ventilated, vagal nerves transected to eliminate nerve reflexes, and exposed to increasing concentrations of nebulized methacholine. Airway resistance was calculated as change over airway resistance after nebulized PBS. IL5tg mice (squares, n=5) had reduced airway responsiveness to methacholine compared with WT (circles, n=5) and (-)Eos (triangles, n=6) mice.

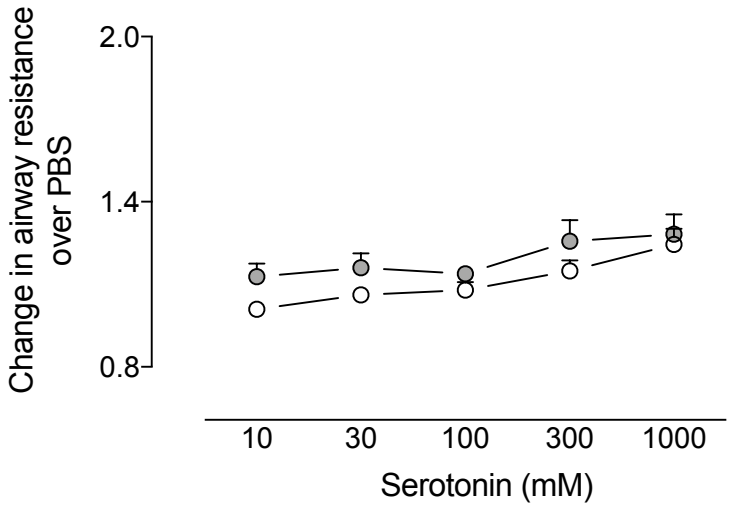

Figure 23. Gallamine did not potentiate reflex bronchoconstriction in WT mice. Inhibition of M_2 muscarinic receptors with gallamine (1 mg/kg, i.v.; grey; n=6) did not increase serotonin-induced reflex bronchoconstriction in untreated WT mice (white; n=8)

D. Discussion

Early life respiratory insults increase airway hyperreactivity and permanently alter airway innervation[526,529,533-536]. My data show that parasympathetic ganglia comprise a complicated network of neurons that continue to develop after birth and respond to eosinophilic inflammation. Eosinophils are recruited to the lung after birth normally and this is increased by allergen exposure[538-540]. I have shown that once in the lungs, eosinophils suppress parasympathetic ganglia expansion and neurotransmitter heterogeneity, which may contribute to lifelong airway hyperreactivity[206] (and figure 25b). IL5tg mice exhibit increased reflex bronchoconstriction (figure 25b), thus it was surprising that they also have reduced smooth muscle contractility. Increased reflex bronchoconstriction may cause chronic acetylcholine release from airway nerves, leading to downregulation of M_3-muscarinic receptor expression and decreased smooth muscle contractility[610].

Why parasympathetic ganglia contain rare neurons that express substance P, nNOS, and tyrosine hydroxylase is not clear. Parasympathetic ganglia develop from neural crest cells[510,511]. A subset of developing neural crest cells co-express sensory neuropeptides and choline acetyltransferase or substance P and tyrosine hydroxylase[611-613], thus these rare parasympathetic neurons may reflect an immature population of neurons. Neural crest cells do not divide further after expressing substance P[612], which may explain why substance P positive neurons described here did not change after birth. Subpopulations of enteric neural crest

cells transiently express tyrosine hydroxylase during development[614] and a similar phenomenon may occur in airway parasympathetic ganglia. Postnatal maturation and terminal differentiation of neurons may explain why tyrosine hydroxylase positive neurons decreased after birth.

Similarly, how these rare neurons function within the broader ganglia network to regulate airway hyperreactivity is unknown. Substance P increases cholinergic bronchoconstriction[288] by potentiating synaptic transmission through ganglia[289,290]. Nitric oxide also regulates neuron excitability[615], thus it is possible that these neurons function as regulatory neurons to control ganglion neurotransmission. Tyrosine hydroxylase expressing neurons found in parasympathetic ganglia of other organs do not contain detectable catecholamines[605], thus it is unclear whether tyrosine hydroxylase expression described here is functional. These neurons are also morphologically distinct from small intensely fluorescent cells that have been detected previously in parasympathetic ganglia and that also express tyrosine hydroxylase[605], thus future studies dissecting the functional role of tyrosine hydroxylase expression in parasympathetic ganglia will need to account for these two cell types.

Co-expression of substance P and choline acetyltransferase (figure 19) suggests that neurotransmitter expression does not identify distinct neuronal lineages. Culture of gut-derived neural crest cells with GDNF or bone morphogenetic protein-2 can induce differentiation of nNOS or tyrosine hydroxylase expressing

neurons from the same precursor population[616], suggesting molecular and developmental pathways exist for neurons to express multiple phenotypes depending on target-derived cues. Identifying innervation targets of parasympathetic neurons that express substance P, nNOS, or tyrosine hydroxylase may illuminate why these rare neurons exist and how they differ from neighboring neurons within ganglia.

Whether postnatal parasympathetic ganglia expansion and neurotransmitter heterogeneity occurs in humans or after relevant environmental stimuli are important questions. Human airway ganglia express substance P[227], suggesting airway ganglia heterogeneity exists in humans. Experiments exposing mice to allergen, ozone, or viral infection will answer if heightened postnatal eosinophil influx into lungs causes suppression of ganglia expansion similar to overexpression of IL-5.

In summary, my data show that show that parasympathetic ganglia continue to develop after birth, contain rare neurons expressing substance P, nNOS, and tyrosine hydroxylase, and respond to eosinophilic inflammation (Figure 24). Basic, fundamental questions of why neurotransmitter heterogeneity exists within parasympathetic ganglia, what rare neurons innervate and what signals terminate differentiation remain to be explored. These experiments are ongoing in the Jacoby laboratory.

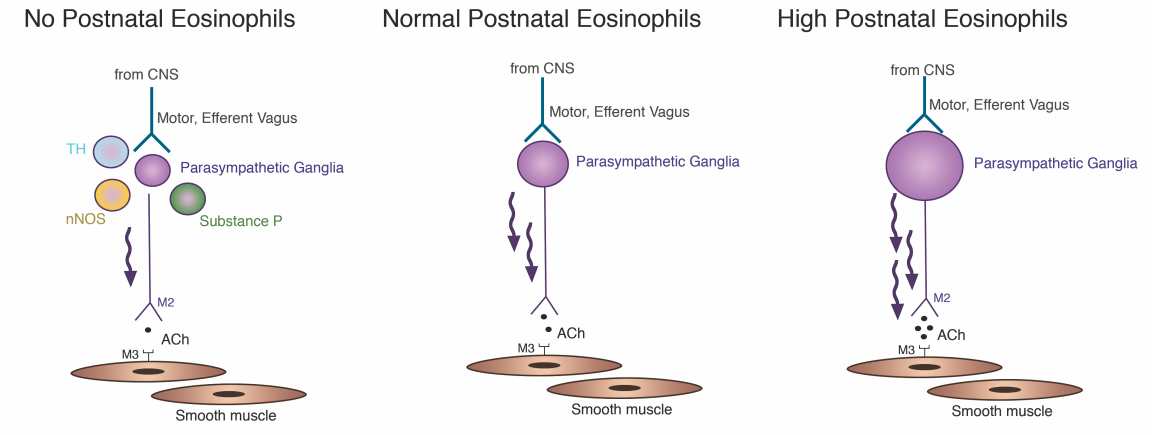

Figure 24. Schematic of chapter 4 results. Parasympathetic ganglia continue to develop after birth, increasing in number and changing expression of substance P, nNOS, and tyrosine hydroxylase. If eosinophils are deleted, parasympathetic ganglia increase, with maximal heterogeneity in neurotransmitter expression. If eosinophils increase, parasympathetic ganglia do not expand, instead forming large aggregate ganglia with few neurons expressing substance P, nNOS, or tyrosine hydroxylase.

E. Methods

E1. Statistical analysis

Data graphed as mean ± SEM and analyzed using GraphPad Prism 7. Number of animals used denoted in figure legends. Ganglia and neuron data analyzed with a one-way ANOVA with Sidak's posttest and reported *p* values represent post-test statistical value. Airway resistance data were analyzed with a repeated-measures two-way ANOVA. Reported *p* values represent interaction term of two-way ANOVA (genotype or treatment x serotonin dose response).

CHAPTER 5. Eosinophils regulate substance P and CGRP expression and degradation

A. Abstract

Stimulation of airway sensory nerves causes local reflex release of substance P and CGRP, two neuropeptides with remarkably similar pathways regulating expression, release, and degradation, but with opposing effects on airway physiology. Both neuropeptides increase with allergen sensitization and exposure, but whether inflammatory phenotypes differentially regulate substance P and CGRP expression is not clear. Similarly, if both neuropeptides increase with allergen exposure, it is unclear whether substance P and CGRP functionally antagonize each other. Here I show that eosinophilic airway inflammation increases substance P, in both lavage and airway nerves, while neutrophilic airway inflammation increases lavage, but not nerve, substance P. Neutrophilic airway inflammation also increases nerve and lavage CGRP, however no change in airway CGRP occurs with eosinophilic inflammation. Eosinophils increase airway hyperreactivity, in part by inhibiting neutral endopeptidase and suppressing neuropeptide degradation, which does not occur in mice with neutrophilic inflammation. In contrast to airway hyperreactivity, vascular leakage after inhaled serotonin is greater in mice with neutrophilic inflammation compared to mice with eosinophilic inflammation. These data show that inflammatory response to allergen differentially regulates substance P and CGRP expression,

which correlates with inverse effects on airway hyperreactivity and vascular leakage.

B. Introduction

Airway sensory nerves co-express substance P and CGRP and innervate airway epithelium, vasculature, smooth muscle, and parasympathetic ganglia[219-223,313,315,317,318]. Similar nerve stimuli induce substance P and CGRP secretion[237-239,241,243,320-322,324,325], but substance P and CGRP have opposing effects on airway function. Substance P increases mucus secretion[261-263] and vascular leakage[260,264,265], while CGRP causes vasodilation[345,346]. Substance P recruits[271-273] and activates immune cells, stimulating immune cell secretion of inflammatory cytokines[276-278], oxidized lipids[279], and granule proteins[280]. In contrast, CGRP suppresses inflammation by inhibiting macrophage[351] and dendritic cell antigen presentation[352-354], and promoting IL-10 secretion and development of tolerogenic T regulatory cells[352]. Substance P directly contracts smooth muscle[283-286] and increases cholinergic bronchoconstriction[288] by potentiating synaptic transmission through ganglia[289,290] and facilitating acetylcholine release[292-294]. CGRP, however, blocks serotonin[361] or substance P-induced smooth muscle contraction[362] and suppresses allergen-induced airway hyperreactivity[342], potentially by hyperpolarizing airway ganglia[318] and inhibiting acetylcholine release[363,364].

Individuals with asthma have elevated substance P in airway lavage fluid[295,296], airway nerves[297], and peripheral blood[298] compared to individuals without asthma. Allergen exposure increases airway nerve substance P expression[299-303] and blocking substance P receptors prevents allergen-induced airway hyperreactivity[306] in animal models of asthma. However, allergen sensitization and exposure also increases airway lavage CGRP[341], which inhibits airway hyperreactivity[342]. Substance P and CGRP expression increases in response to IL-1β[233,337,338] and TNFα[234,235,336], inflammatory cytokines that are elevated after allergen exposure. Here I tested whether eosinophilic vs. neutrophilic inflammatory phenotypes differentially regulate substance P and CGRP expression to influence airway hyperreactivity, inflammation, and vascular leakage.

C. Results

C1. Eosinophils are required for airway hyperreactivity

WT and eosinophil-deficient (-)Eos mice were sensitized to HDM on protocol day 0 and 1 and then exposed to HDM on days 14-17. On day 18, mice were sedated, mechanically ventilated, and exposed to increasing concentrations of inhaled serotonin to cause reflex bronchoconstriction. Allergen-naïve mice with airway eosinophilia (IL5tg) were also used to test if eosinophils increased reflex bronchoconstriction in the absence of allergen exposure.

HDM exposure in WT mice (Figure 25a) caused airway hyperreactivity compared to vehicle-exposed mice (Figure 25a). Airway eosinophilia in IL5tg mice increased reflex bronchoconstriction (Figure 25b) comparable to HDM exposed WT mice. Deletion of eosinophils prevented serotonin-induced bronchoconstriction (Figure 25c), demonstrating that eosinophils are required for airway hyperreactivity after HDM exposure.

C2. HDM exposure causes eosinophilic inflammation in WT mice and neutrophilic inflammation in (-)Eos mice.

HDM exposure increased airway total inflammatory cells in WT mice (Figure 23a), which consisted mostly of eosinophils (Figure 26b). Airway inflammation increased less after HDM exposure in (-)Eos mice compared with WT mice (Figure 26a) and consisted mostly of neutrophils (Figure 26c) in these mice.

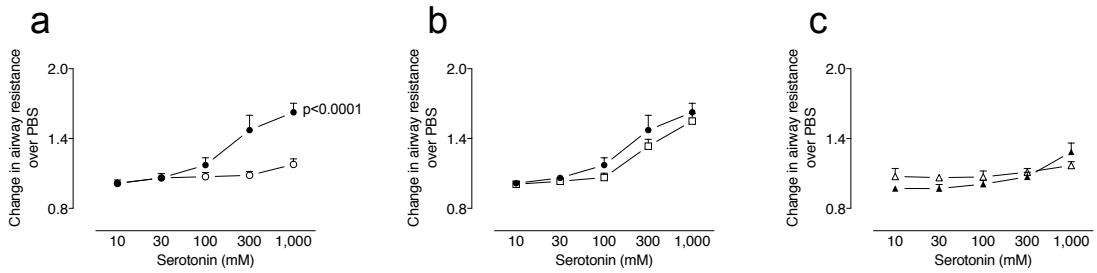

Figure 25. Eosinophils were required for airway hyperreactivity. (a) WT mice develop airway hyperreactivity after HDM sensitization and exposure (black circles, n=8) compared with vehicle exposed mice (white circles, n=8). (b) Airway hyperreactivity in HDM exposed WT mice (black circles, n=8) was similar to increased reflex bronchoconstriction in allergen-naïve IL5tg mice (white squares, n=8). (c) Depleting eosinophils prevents airway hyperreactivity after HDM exposure (white triangles, vehicle exposed (-)Eos mice, n=8; black triangles, HDM exposed (-)Eos mice, n=8).

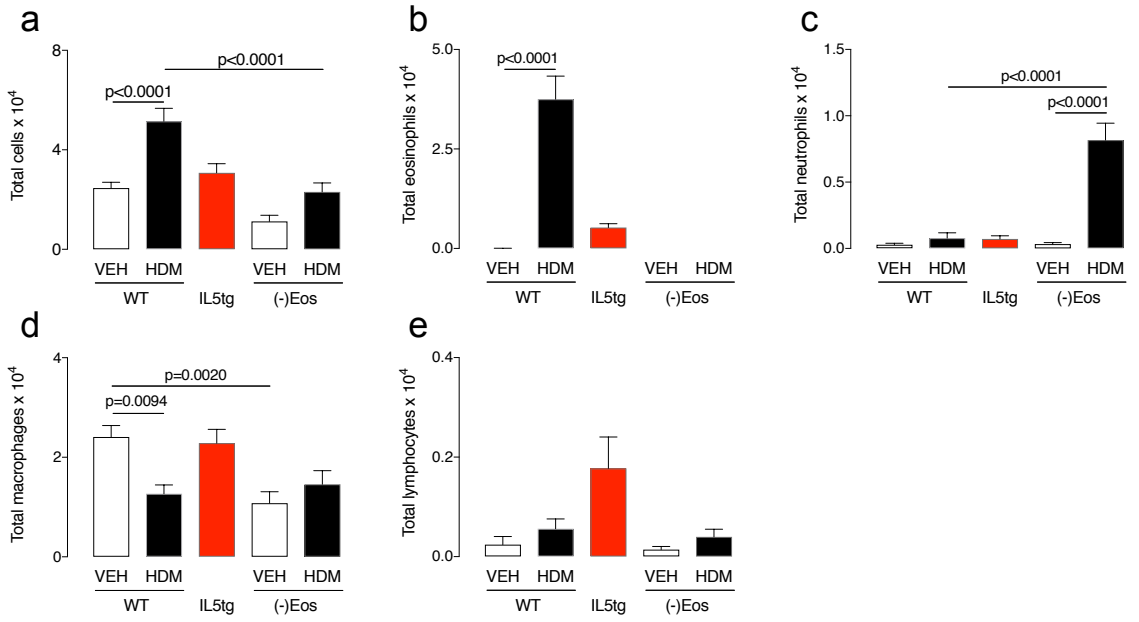

Figure 26. HDM exposure caused eosinophilic inflammation in WT mice and neutrophilic inflammation in (-)Eos mice. (a) Total lavage inflammatory cells increased in WT mice with HDM exposure and this was decreased in (-)Eos mice. (b) Lavage eosinophils increased with HDM exposure in WT mice. (c) HDM exposure increased airway neutrophils (-)Eos mice. (d) Macrophages declined with HDM exposure in WT mice, but did not change in (-)Eos mice. (e) Lymphocytes tended to increase with HDM exposure independent of eosinophils. N=8 all groups.

C3. Eosinophilic inflammation increases substance P while neutrophilic inflammation increases CGRP.

To test which inflammatory phenotypes increase substance P and CGRP, airways were lavaged with PBS and neuropeptides measured with ELISA. HDM exposure increased substance P in both WT and (-)Eos mice (Figure 27a), demonstrating that lavage substance P increases with allergen exposure independent of inflammatory phenotypes. Similarly, IL5tg mice had more substance P than vehicle-exposed WT mice, demonstrating that eosinophil influx into the lungs is sufficient for induction of substance P without allergen exposure. Lavage substance P could represent lung or immune cell derived substance P, thus whole-mount tracheas were stained for substance P and PGP9.5 (a pan-neuronal marker), optically cleared, and imaged with confocal microscopy. 3D dimensional nerve models of epithelial, subepithelial, and smooth muscle nerves were created and percent of total nerves expressing substance P calculated. Airway nerve substance P increased with HDM exposure in WT mice and with airway eosinophilia in IL5tg mice, but did not increase with HDM exposure in (-)Eos mice (Figure 27b). These data demonstrate that eosinophilic inflammation increases substance P in both nerves and lavage fluid, while neutrophilic inflammation increases substance P only in lavage fluid.

In contrast to substance P, HDM exposure increased lavage CGRP only in (-)Eos mice (Figure 28a) and CGRP was detectable in vagal ganglia neurons only from (-)Eos mice (Figure 28b). These data demonstrate that airway inflammatory

phenotypes differentially increase nerve neuropeptide expression, with eosinophilic inflammation increasing substance P and neutrophilic inflammation increasing CGRP.

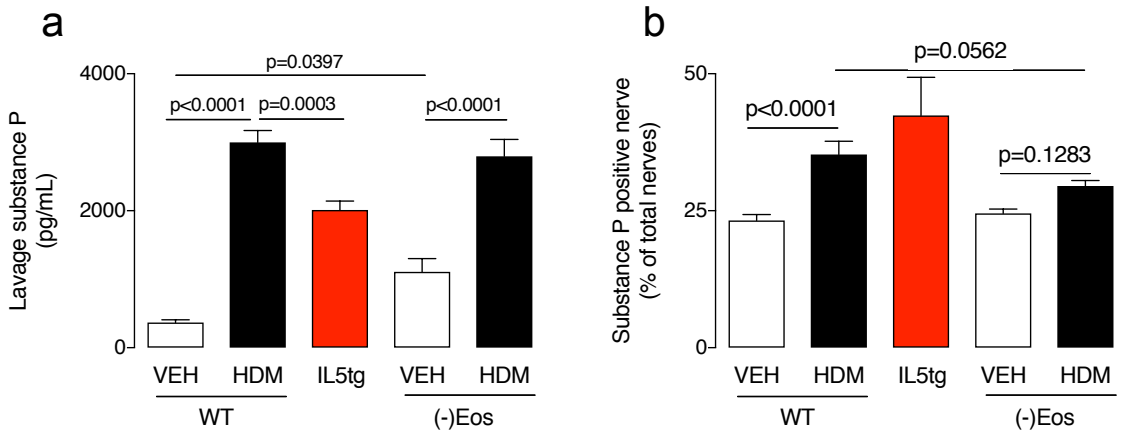

Figure 27. Inflammation increases airway substance P. (a) Vehicle exposed (-)Eos mice had more substance P than vehicle-exposed WT mice. Lavage substance P increased with HDM exposure in both WT and (-)Eos mice. (b) Percentage of airway nerves in whole-mount tissues that expressed substance P increased with HDM exposure in WT mice, but not in (-)Eos mice. For lavage analysis, n=8, except IL5tg, n=10. For nerve analysis, n=8, except IL5tg, n=7.

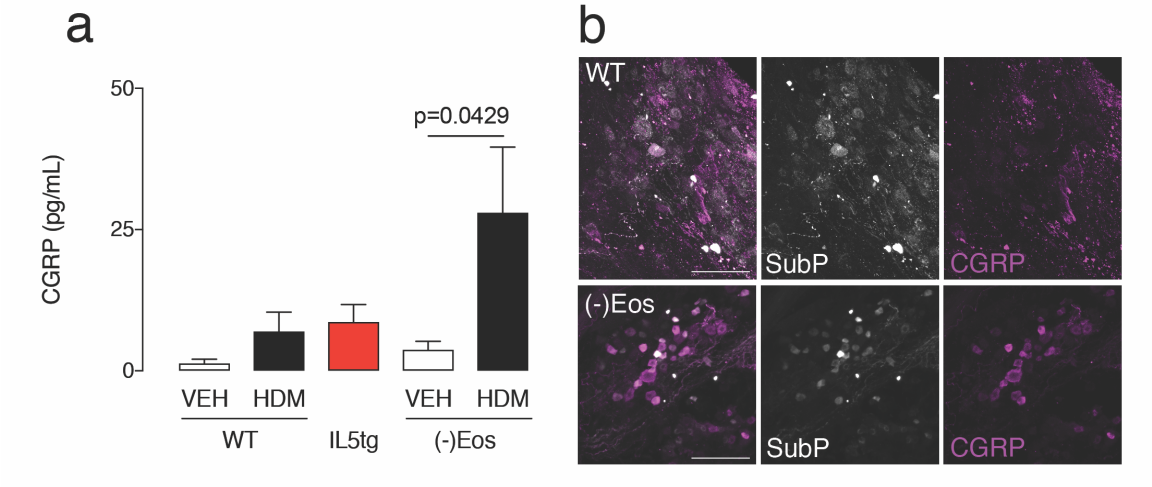

Figure 28. Neutrophilic inflammation increased airway CGRP. (a) CGRP increased in airway lavage in (-)Eos mice exposed to HDM, but less so in HDM exposed WT mice. N=8 all groups. (b) vagal ganglia neurons expressed abundant CGRP in (-)Eos mice, but not in WT mice. Scale bar = 75 μm for (-)Eos and 50 μm for WT.

C4. Eosinophils suppress neutral endopeptidase activity

Neutral endopeptidase degrades both substance P and CGRP[333] and competition for degradation may influence relative effects on airway physiology. Inhibition of substance P degradation by neutral endopeptidase can also limit CGRP function by causing mast cell degranulation and degradation of CGRP by mast cell tryptase[350]. To test whether inflammatory phenotypes differentially inhibit neuropeptide degradation, HDM and vehicle exposed mice were sedated, mechanically ventilated, and administered phosphoramidon to inhibit to neutral endopeptidase before measuring responses to inhaled serotonin.

Inhibition of neutral endopeptidase potentiated reflex bronchoconstriction in vehicle-exposed WT mice (Figure 29a), demonstrating that neutral endopeptidase functions at baseline to limit airway response to neuropeptides. Inhibition of neutral endopeptidase did not increase airway hyperreactivity further in HDM exposed WT mice (Figure 29b) or reflex bronchoconstriction in IL5tg mice (Figure 29c), suggesting eosinophilic inflammation inhibits neutral endopeptidase. In contrast, inhibition of neutral endopeptidase potentiated airway reactivity in both vehicle and HDM exposed (-)Eos mice (Figure 29d and 29e), demonstrating that neutrophilic inflammation does not inhibit neutral endopeptidase. Mixing airway lavage fluid with a fluorescent neutral endopeptidase substrate *in vitro* and measuring change in fluorescence confirmed that HDM exposure suppresses endogenous neutral endopeptidase activity in WT mice, but not in (-)Eos mice (Figure 29f)

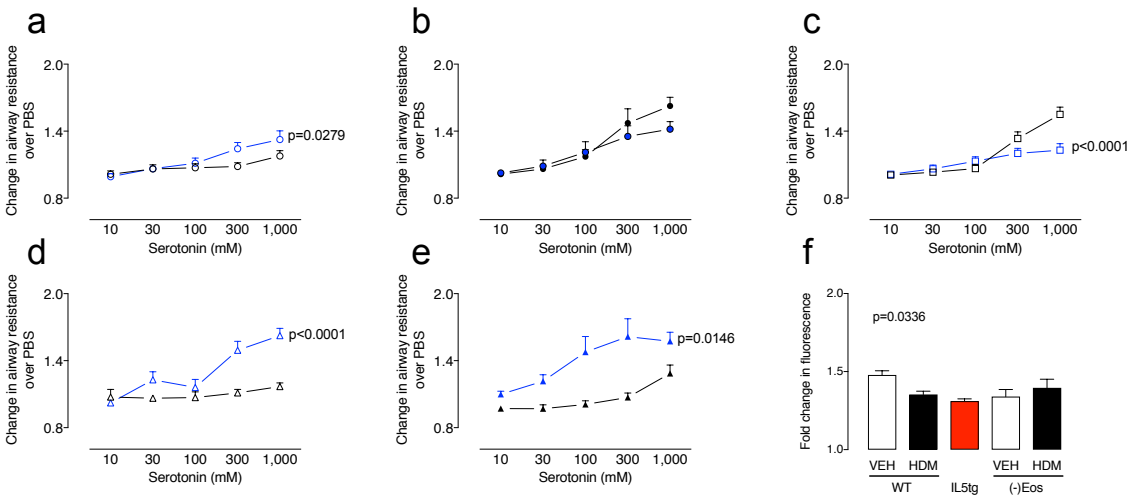

Figure 29. Eosinophils suppressed neutral endopeptidase activity. Vehicle and HDM exposed mice were pretreated with phosphoramidon to inhibit neutral endopeptidase activity and airway responses to inhaled serotonin measured. (a) phosphoramidon potentiated airway reactivity in vehicle exposed WT mice. (b) phosphoramidon did not potentiate airway hyperreactivity in HDM exposed WT mice. (c) Phosphoramidon suppressed airway hyperreactivity in IL5tg mice. (d) Phosphoramidon potentiated airway reactivity in vehicle-expsed (-)Eos mice. (e) Phosphoramidon potentiated airway reactivity in HDM exposed (-)Eos mice. (f) Endogenous neutral endopeptidase activity decreased with HDM exposure in WT mice, but not in (-)Eos mice. N=8 for all groups, except (-)Eos vehicle exposed mice treated with phosphoramidon, n=6; IL5tg mice treated with phosphoramidon, n=7; and IL5tg panel f, n=6.

C5. Neutrophilic inflammation increases vascular leakage

Activation of sensory nerves causes reflex edema and vascular leakage. To test whether neuropeptide expression in eosinophilic vs. neutrophilic inflammation differentially correlated with nerve-evoked vascular leakage, mice were administered Evan's Blue dye via the external jugular vein and dye extravasation measured in the trachea before and after inhaled serotonin (in independent cohorts of mice).

At baseline, dye extravasation was the same between WT and (-)Eos mice (Figure 30a). HDM exposure did not increase dye extravasation in WT or (-)Eos mice. Inhaled serotonin increased dye extravasation in all groups, but vascular leakage was greatest in HDM exposed (-)Eos mice and significantly more compared to HDM exposed WT mice (Figure 30b).

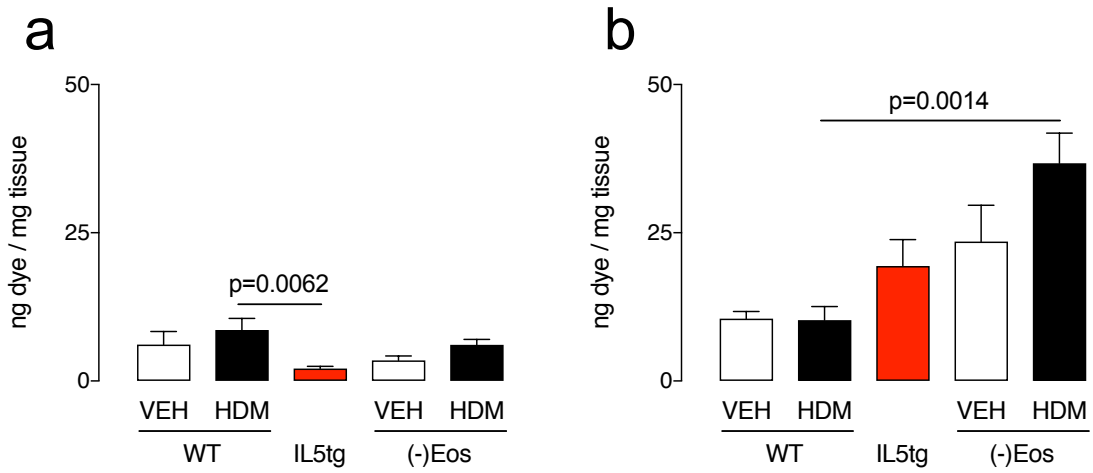

Figure 30. Neutrophilic inflammation increased vascular leakage. Evans blue dye extravasation was measured in trachea from mice at baseline (a) and after exposure to inhaled serotonin (b). (a) Vascular leakage was the same between WT and (-)Eos mice and did not change with HDM exposure (WT VEH n=4, HDM n=5; IL5tg n=6; (-)Eos VEH n=5, HDM n=3). (b) Vascular leakage increased in all mice exposed to inhaled serotonin. Dye extravasation was greater in HDM exposed (-)Eos mice compared with HDM exposed WT mice (WT VEH n=4, HDM n=6; IL5tg n=5; (-)Eos VEH n=5, HDM n=6).

C6. Neuropeptide receptor agonists and antagonists

The above studies show only a correlation between neuropeptide expression and different responses in airway hyperreactivity and vascular leakage. To test the direct effect of substance P on airway hyperreactivity, neutral endopeptidase and angiotensin converting enzyme (which also degrades substance P) were inhibited with phosphoramidon and captopril, respectively, and airway responses to i.v. substance P measured. Substance P did not increase airway resistance in vehicle exposed (Figure 31a), HDM exposed WT mice (Figure 31a and figure 31b), or HDM exposed (-)Eos mice (Figure 31b). Substance P is unstable in solution, thus a nonpeptide NK1 agonist was also tested. Intravascular injection of GR73632 also did not increase airway resistance in a vehicle exposed WT mouse (Figure 31c). Thus, an opposite approach using a NK1 antagonist was used. Inhibition of NK1 receptors in HDM exposed mice before inhaled serotonin unexpectedly worsened airway hyperreactivity (Figure 12a), which contrasts with previous reports in guinea pigs asthma models[306]. To test whether this was specific to NK1 receptors or whether other tachykinin receptors also worsen airway hyperreactivity in mice, NK2 receptors were blocked in HDM exposed WT mice before serotonin. However, the NK2 antagonist is hydrophobic and both solvents tested suppressed airway hyperreactivity (Figure 32). Due to technical difficulties, these experiments were not continued.

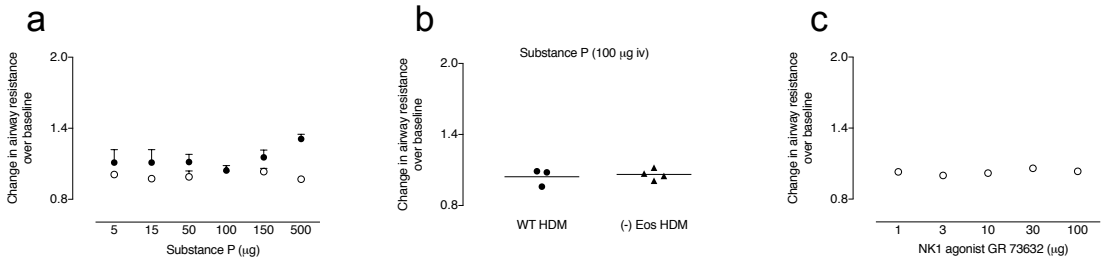

Figure 31. Neurokinin-1 agonists did not increase airway resistance. (a) Substance P administered iv to vehicle exposed (white circles, n=1 full dose response curve; n=2 all doses except 500 μg; n=6 only 100 μg dose) and HDM exposed (black circles; n=2 full dose response curve; n=4 only 100 μg dose) mice did not increase airway resistance. (b) Substance P administered iv to HDM exposed WT (black circles) and (-)Eos mice (black squares) did not increase airway resistance. (c) GR73632, an NK1 agonist, administered iv did not increase airway resistance in a vehicle exposed WT mouse.

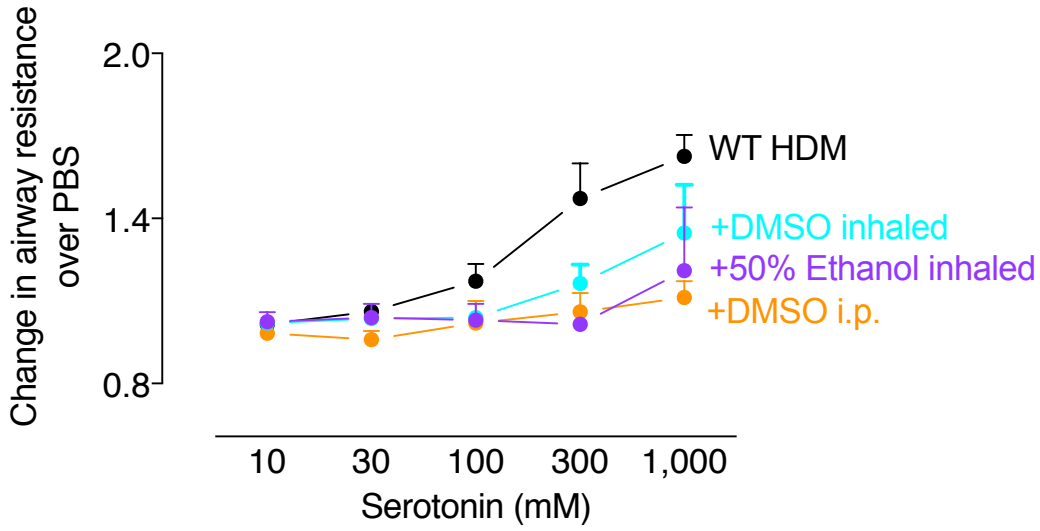

Figure 32. Solvents for neurokinin-2 antagonists suppressed airway hyperreactivity. DMSO and ethanol suppressed airway hyperreactivity in HDM exposed WT mice (WT HDM, n=8; inhaled ethanol control, n=2; inhaled DMSO control, n=2; i.p. DMSO control, n=3).

C7. Chronic house dust mite treatment increases inflammation and neuropeptide expression

Asthma is a chronic inflammatory disease and increased neuropeptide expression after acute allergen exposure may not reflect what occurs with ongoing airway inflammation. Thus, mice were challenged with HDM 5 days per week and airway inflammation and neuropeptide expression measured after 8 weeks. HDM exposure increased airway inflammation in WT mice, but not in (-)Eos mice (Figure 33a). Inflammation after chronic house dust mite consisted mostly of eosinophils in WT mice (Figure 33b), but neutrophils also increased (Figure 33c). The small increase in airway inflammation in (-)Eos mice with chronic HDM exposure consisted solely of neutrophils (Figure 33c), similar to acute HDM exposure. Macrophages were more abundant in WT than (-)Eos mice and did not change with chronic HDM exposure (Figure 33d). Lymphocytes also did not change with chronic HDM exposure (Figure 33e).

Substance P and CGRP were measured in airway lavage fluid from mice chronically exposed to HDM. Similar to acute HDM exposure, substance P increased in both WT and (-)Eos mice with chronic HDM exposure (Figure 34a), while CGRP increased only in (-)Eos mice (Figure 34b). These data demonstrate that neuropeptide expression increases with both acute and chronic airway inflammation.

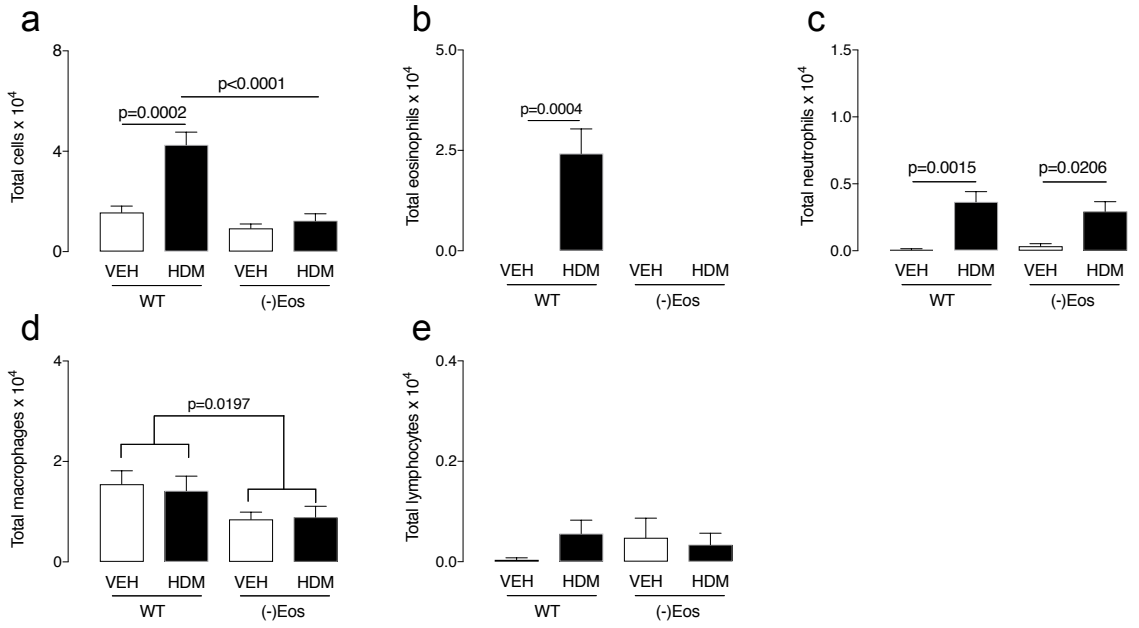

Figure 33. Chronic house dust mite exposure increased airway inflammation. (a) Total inflammatory cells increased in WT, but not (-)Eos mice, exposed to inhaled HDM or vehicle for 8 weeks. (b) Eosiophils increased with chronic HDM exposure. (c) Neutrophils increased with chronic HDM exposure in both WT and (-)Eos mice. (d) WT mice had more lavage macrophages than (-)Eos mice. Chronic HDM exposure did not change airway macrophages. (e) Lymphocytes did not change in WT or (-)Eos mice after chronic HDM exposure. N=5 all groups.

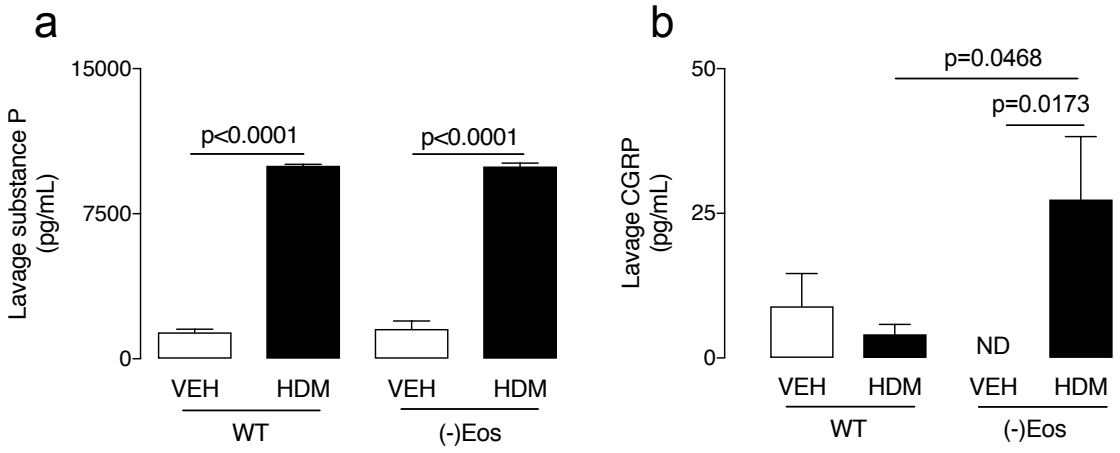

Figure 34. Chronic HDM exposure increased airway substance P and CGRP. (a) Lavage substance P increased in HDM exposed WT and (-)Eos mice. (b) Lavage CGRP increased only in (-)Eos mice with chronic HDM exposure. N=5 all groups, except CGRP in (-)Eos HDM, n=4.

D. Discussion

Asthma is a heterogeneous disease with different underlying mechanisms and variable patient response to therapies. The need to identify pathobiologic mechanisms with clinical utility led to the creation of asthma phenotypes and endotypes, which marries molecular biomarkers with clinical characteristics, lung physiology, inflammation, histopathology, and treatment response[3] in hope of creating improved, personalized treatment options. My data show that different inflammatory phenotypes, eosinophilic versus neutrophilic inflammation, promote different pathologic features of asthma. Eosinophilic inflammation causes airway hyperreactivity and increases nerve substance P, while neutrophilic inflammation causes vascular leakage and increases nerve CGRP.

Expression of substance P in different tissue compartments is important for understanding its role in airway hyperreactivity. Lavage substance P was the same between HDM exposed WT and (-)Eos mice, and vehicle-exposed (-)Eos mice had more lavage substance P than vehicle-exposed WT mice, yet (-)Eos were not hyperreactive. In contrast, HDM exposure increased nerve substance P only in WT mice. Substance P increases cholinergic bronchoconstriction[288] by potentiating synaptic transmission through ganglia[289,290] and facilitating acetylcholine release[292-294], thus nerve-specific and not airway lumen substance P may potentiate airway hyperreactivity. Conversely, CGRP inhibits substance P induced smooth muscle contraction[362]. Thus, increased CGRP expression in

(-)Eos mice may block substance P signaling and prevent airway hyperreactivity. If that occurred, then neuropeptide balance may be more important than compartment specific expression of a single neuropeptide.

It was surprising that blocking neurokinin-1 receptors worsened airway hyperreactivity in HDM exposed WT mice, since inhibiting neurokinin-1 receptors in guinea pig models of asthma decreases airway hyperreactivity[306]. Activation of afferent sensory nerves can elicit peripheral nerve reflexes to cause NK1-dependent, NANC relaxation of airway smooth muscle[165]. Substance P also relaxes pre-contracted airway smooth muscle via epithelial release of prostaglandins[586,587], however this occurs in both mice and guinea pigs and thus not explain why blocking NK1 has species-specific effects. Different species, choice of allergen, *in vitro* versus *in vivo* model systems, and epithelial / luminal versus nerve substance P expression make comparing studies difficult.

Neutral endopeptidase and angiotensin-converting enzyme[244] degrade substance P and inhibition of either enzyme potentiates airway responses to substance P[245-248]. Suppression of neutral endopeptidase activity contributes to airway hyperreactivity and occurs after viral infection[253], allergen[254,255] and oxidant exposure[256,257]. My data show that eosinophilic inflammation suppresses neutral endopeptidase activity, but not neutrophilic inflammation. Eosinophils express eosinophil peroxidase and may suppress neutral endopeptidase by creating hypobromous acid. However, neutral endopeptidase is also suppressed

by hypochlorous acid[257]. Thus, if oxidants were responsible for neutral endopeptidase inhibition, phosphoramidon should have potentiated airway reactivity in mice with either eosinophilic or neutrophilic inflammation. HDM exposed WT mice had more inflammation than (-)Eos mice, so perhaps the relative magnitude of inflammation was important for suppression of neutral endopeptidase in WT mice. Further studies will be needed to clarify why neutral endopeptidase was inhibited in HDM exposed WT mice and not in HDM exposed (-)Eos mice.

Neutral endopeptidase degrades other neurotransmitters, such as neurokinin A[258] and bradykinin[259], thus potentiation of airway responses after inhibition of neutral endopeptidase cannot solely be attributed to increased substance P. Several possibilities exist. Neurokinin A more potently causes bronchoconstriction than substance P[285], thus increased airway hyperreactivity in HDM exposed WT mice and phosphoramidon treated (-)Eos mice may reflect increased neurokinin A. Antibodies frequently cross-react with both substance P and neurokinin A, thus dissecting the biology of these two tachykinins requires use of antagonists. However, I was unable to test this due to technical difficulties with solvents for a NK2 receptor antagonist. Another possibility is that the balance between substance P and CGRP was disrupted in (-)Eos mice. Neutral endopeptidase more efficiently degrades substance P[333], and increased substance P can cause mast cell degranulation and subsequent degradation of CGRP[350]. Thus, potentiation of airway reactivity in (-)Eos mice may reflect a loss

of protective CGRP. Measuring CGRP before and after phosphoramidon, or using NK1 antagonists in (-)Eos treated with phosphoramidon, may further clarify these questions.

Vascular leakage contributes to airway edema and airflow obstruction in asthma. CGRP can potentiate substance P-mediated edema[347-349], presumably due to vasodilatation and increased local blood volume. Increased vascular leakage in HDM exposed (-)Eos mice may reflect synergism between elevated airway CGRP and substance P in these mice.

In summary, my data show that eosinophilic and neutrophilic inflammation promotes distinct pathologic features of asthma (Figure 35). Eosinophils caused airway hyperreactivity and increased substance P expression, while neutrophils caused vascular leakage and increased CGRP expression. Inflammatory phenotyping of patients with asthma may thus guide therapy choice to selectively relieve asthma symptoms.

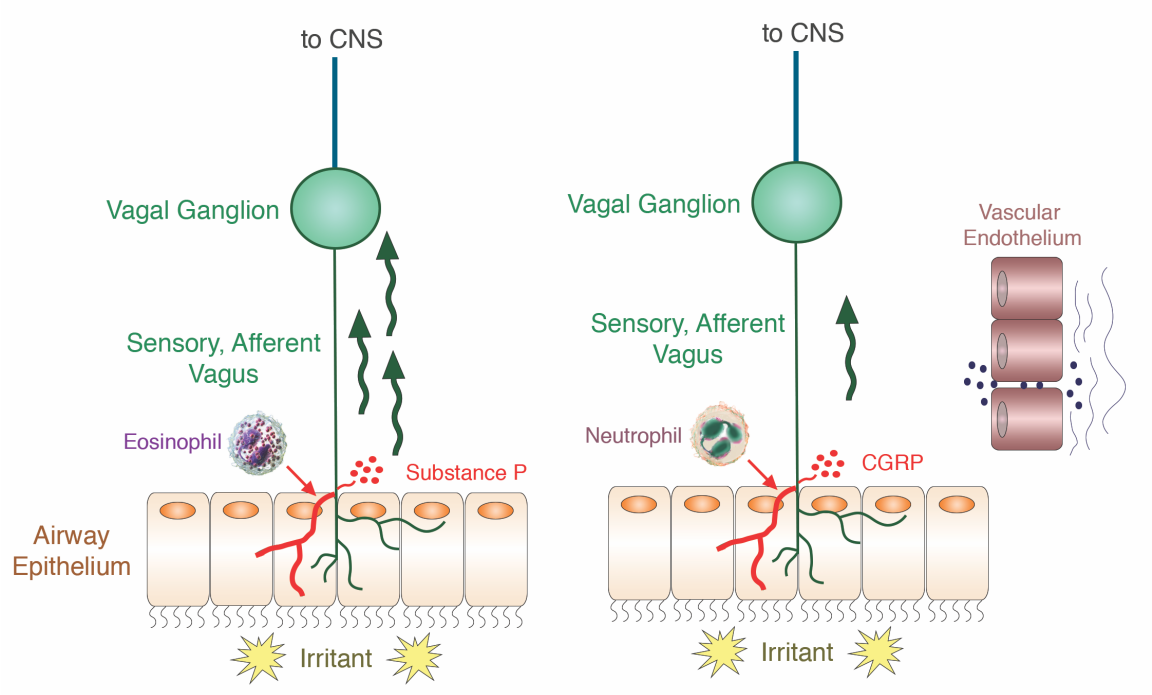

Figure 35. Schematic of chapter 5. Eosinophilic and neutrophilic airway inflammation promote different pathologic features of asthma and increase expression of different neurotransmitters. Eosinophilic inflammation increases expression of substance P and causes airway hyperreactivity. Neutrophilic inflammation increases expression of CGRP, does not cause airway hyperreactivity, but does causes vascular leakage. Eosinophil adapted from Wikipedia.org. Neutrophil adapted from eosinophil.

E. Methods

E1. Evan's Blue Dye Extravasation

For baseline measurements Evan's Blue extravasation measurements, mice were euthanized with pentobarbital, a vertical adbdominal incision made and the descending aorta transected. Mice were perfused with PBS via the right ventricle. The trachea was dissected free of the lungs, weighed, and placed into 1 mL of formamide for 48 hours. After 48 hours, the trachea was removed, 100 µL of the solution placed in a 96 well plate, and absorbance (620nm) measured. For serotonin-induced extravasation, mice were placed on the ventilator and exposed to increasing concentrations of inhaled serotonin as decribed in the general methods section. After physiology experiments, tissues were collected and analyzed as described for baseline samples.

E2. Statistics

Data graphed as mean ± SEM and analyzed using GraphPad Prism 7. Number of animals used denoted in figure legends. Airway resistance data were analyzed with a repeated-measures two-way ANOVA. Reported *p* values represent interaction term of two-way ANOVA (genotype or treatment x serotonin dose response). All other data comparing WT and (-)Eos mice were analyzed with a two-way ANOVA with Sidak's posttest and reported *p* values represent post-test statistical value. IL5tg and HDM exposed WT mice were compared with a two-tailed, unpaired t-test.

CHAPTER 6. Summary and Conclusions

Reflex bronchoconstriction is a normal physiologic process (Figure 1), likely designed to protect the airways from inhaled substances. Reflex bronchoconstriction is heightened in individuals with asthma[154] and consists of both persistent responsiveness at baseline and acute hyperreactivity due to airway inflammation[4] (Figure 2). Airway hyperreactivity in patients with asthma is suppressed by inhaled steroids, but seldom to the range of individuals without asthma[5], suggesting permanent structural changes of airway tissue contribute to persistent hyperresponsiveness.

Airway reactivity varies at birth naturally[90], suggesting *in utero* programming of baseline airway responses. Parental asthma increases infant airway reactivity[92] and the risk of their child developing asthma[83,84]. Genetic inheritance of risk alleles and shared environments explain only part of this risk since maternal asthma imposes a greater risk for childhood asthma than paternal disease[85]. I hypothesized that maternal allergen exposure causes structural and functional changes in airway nerves of offspring that are mediated by eosinophils and lead to airway hyperreactivity.

My data demonstrate that maternal and fetal inflammatory responses increase lung innervation (Figure 16), enhancing baseline airway responses and airway inflammation after allergen exposure later in life. These data support my hypothesis. My data are the first to demonstrate a mechanistic link between

maternal asthma and offspring airway hyperreactivity, driven by structural changes in airway nerves. My data also identify IL-5 as a therapeutic target to prevent airway hyperreactivity in offspring born to mothers with asthma. I postulate that increased lung innervation in offspring of mothers exposed to HDM or with elevated IL-5 increase baseline airway responsiveness and sets the stage for postnatal allergen sensitization and inflammatory cell recruitment to cause airway hyperreactivity. This is important because heightened airway responses in early infancy predict later development of asthma in humans[94].

My data also demonstrate that fetal eosinophils increase airway epithelial nerve growth during gestation in response to maternal inflammation, uncovering a novel role for eosinophils in neurodevelopment. Airway epithelial nerve length and branching is increased in IL-5 transgenic mice with pulmonary eosinophilia[206] and in asthmatics with eosinophilic inflammation[208]. My data suggest these changes occur during development, and predispose to asthma development, as opposed to nerve growth increasing after airway inflammation in adult life. How eosinophils increase epithelial nerve growth remains unknown.

The airway continues to develop after birth and environmental insults during this critical window permanently increase airway innervation and reactivity. Eosinophil dependent nerve growth during gestation was restricted to afferent nerves since parasympathetic ganglia number were not different between offspring born to WT mothers compared with offspring born to IL5tg mothers. However, in the

postnatal period, the role of eosinophils changed. Eosinophils suppressed the postnatal development and expansion of parasympathetic ganglia (Figure 24). The postnatal plasticity of these ganglia and ability to respond to eosinophilic airway inflammation is a novel discovery. The changing role of eosinophils in structural neurodevelopment suggests that embryonic and postnatal eosinophils are distinct populations with unique effector functions that differentially change nerve development. It is also possible that afferent and efferent nerve development is temporally distinct or that these nerve subtypes respond differently to inflammatory insults. These ideas remain to be tested. Similarly, eosinophil dependent suppression of parasympathetic ganglia number is only correlated with airway hyperreactivity and future work will need to test how parasympathetic ganglia development influences airway physiology.

Eosinophils changed neurotransmitter expression in addition to nerve structure. My data demonstrate that eosinophils increase substance P in epithelial nerves, both during development and after allergen exposure. Offspring from IL5tg mothers had increased epithelial nerve substance P compared with offspring from WT mothers at baseline (Figure 10), demonstrating that eosinophilic inflammation during development causes permanent and lifelong increases in substance P expression. Nerve growth factor increases neuronal expression of substance P[229]. Nerve growth factor was increased in offspring from IL5tg mothers (Figure 9 and Figure 11), thus it is possible that eosinophils increase nerve growth factor secretion from airway epithelium during development, which

in turn permanently increases nerve substance P. Nerve substance P increases after allergen challenge in WT offspring from WT mothers (Figure 10), however there was no increase in offspring exposed to maternal IL-5, suggesting the population of nerves capable of making substance P is limited and maximally increased by eosinophils during development.

Eosinophils changed neurotransmitter expression in both afferent epithelial nerves and efferent parasympathetic ganglia. Similar to nerve structure, the effect of eosinophils on neurotransmitter expression was different between gestation and postnatal life. Eosinophils increased epithelial nerve substance P expression during development, but suppressed substance P expression in parasympathetic ganglia after birth. Eosinophils also suppressed nNOS and tyrosine hydroxylase expression by parasympathetic ganglia. These data again suggest that embryonic and postnatal eosinophils are distinct populations or that airway afferent and efferent nerves respond differently to eosinophilic inflammation. Heterogeneous neurotransmitter expression in airway parasympathetic ganglia has never been described before. I discovered that these rare neurons expressing substance P, nNOS, and tyrosine hydroxylase exist, exhibit postnatal plasticity, and respond to eosinophilic airway inflammation. The contribution of these rare neurons to airway physiology and the functional significance of reduced neurotransmitter heterogeneity in airway parasympathetic ganglia are ongoing experiments in the Jacoby laboratory.

Eosinophil-nerve interactions are not limited to the airway and my work may translate to other disease states. Maternal HDM exposure is associated with increased risk of atopic dermatitis in children[617], an allergic skin disease with prominent eosinophilia. Intractable itch is a common and debilitating symptom of the disease that is nerve-mediated[618] and dependent upon eosinophils in mice[209] Nerve length, branching, and nerve substance P are also increased in atopic dermatitis and reduced with ablation of eosinophils[207,209]. Thus it is attractive to speculate that eosinophils increase nerve growth in the skin during development similar to the airways and this contributes to atopic dermatitis risk and disease severity.

Eosinophils also reside in healthy and diseased gastrointestinal tissue and interact with gastrointestinal nerves[619,620]. Gastrointestinal nerves secrete eotaxin[619] to actively recruit eosinophils, similar to airway nerves[372], and eosinophil recruitment is associated with increased esophageal nerve sensitivity after oral allergen exposure[621]. These data demonstrate that eosinophil-nerve interactions are conserved across multiple mucosal sites and contribute to pathology in several diseases.

Asthma is a heterogeneous disease with variable patient response to therapies. Understanding how eosinophils change nerve function may ultimately lead to improved, personalized treatment options. In chapter 5, I found that inflammatory phenotypes promote different pathologic features of asthma, with different effects

on airway nerves and airway hyperreactivity (Figure 34). Eosinophilic inflammation causes airway hyperreactivity and increases nerve substance P, while neutrophilic inflammation causes vascular leakage and increases nerve CGRP. Targeted therapies exist for patients with eosinophilic asthma, and my data show that these patients are most likely to have airway hyperreactivity and may benefit from nerve directed therapies. In patients with neutrophilic asthma, my data suggests nerve changes may protect against airway hyperreactivity, and instead these patients may benefit from therapies that limit airway edema. Much work is needed to test these hypotheses and whether my work translates to clinical care of individuals with asthma.

In summary, my data show that maternal asthma increases offspring airway hyperreactivity by increasing lung innervation. Persistent airway hyperreactivity thus results from aberrant nerve development, occurring *in utero* for afferent nerves and after birth for efferent nerves. These changes are dependent on gestational and postnatal eosinophils and represent a therapeutic target to reduce offspring airway hyperreactivity.

CHAPTER 7. References

1 Masoli, M., Fabian, D., Holt, S., Beasley, R. & Global Initiative for Asthma, P. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* **59**, 469-478, doi:10.1111/j.1398-9995.2004.00526.x (2004).

2 GINA. Global Strategy for Asthma Management and Prevention. (2017).

3 Lotvall, J. *et al.* Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol* **127**, 355-360, doi:10.1016/j.jaci.2010.11.037 (2011).

4 O'Byrne, P. M. & Inman, M. D. Airway hyperresponsiveness. *Chest* **123**, 411S-416S (2003).

5 Boulet, L. P. *et al.* Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* **162**, 1308-1313, doi:10.1164/ajrccm.162.4.9910051 (2000).

6 Hoyle, G. W. *et al.* Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. *Am J Respir Cell Mol Biol* **18**, 149-157, doi:10.1165/ajrcmb.18.2.2803m (1998).

7 Leigh, R. *et al.* Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol Biol* **27**, 526-535, doi:10.1165/rcmb.2002-0048OC (2002).

8 Southam, D. S., Ellis, R., Wattie, J. & Inman, M. D. Components of airway hyperresponsiveness and their associations with inflammation and remodeling in mice. *J Allergy Clin Immunol* **119**, 848-854, doi:10.1016/j.jaci.2006.12.623 (2007).

9 Robinson, D. S. *et al.* Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* **326**, 298-304, doi:10.1056/NEJM199201303260504 (1992).

10 Woodruff, P. G. *et al.* T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* **180**, 388-395, doi:10.1164/rccm.200903-0392OC (2009).

11 Romanet-Manent, S. *et al.* Allergic vs nonallergic asthma: what makes the difference? *Allergy* **57**, 607-613 (2002).

12 Humbert, M. *et al.* IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* **154**, 1497-1504, doi:10.1164/ajrccm.154.5.8912771 (1996).

13 Licona-Limon, P., Kim, L. K., Palm, N. W. & Flavell, R. A. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol* **14**, 536-542, doi:10.1038/ni.2617 (2013).

14 Lambrecht, B. N. & Hammad, H. Biology of lung dendritic cells at the origin of asthma. *Immunity* **31**, 412-424, doi:10.1016/j.immuni.2009.08.008 (2009).

15 Trifilieff, A., Fujitani, Y., Coyle, A. J., Kopf, M. & Bertrand, C. IL-5 deficiency abolishes aspects of airway remodelling in a murine model of lung inflammation. *Clin Exp Allergy* **31**, 934-942 (2001).

16 Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I. & Young, I. G. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* **183**, 195-201 (1996).

17 Kung, T. T. *et al.* Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. *Am J Respir Cell Mol Biol* **13**, 360-365, doi:10.1165/ajrcmb.13.3.7654390 (1995).
18 Maes, T., Joos, G. F. & Brusselle, G. G. Targeting interleukin-4 in asthma: lost in translation? *Am J Respir Cell Mol Biol* **47**, 261-270, doi:10.1165/rcmb.2012-0080TR (2012).
19 Brusselle, G. G. *et al.* Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* **24**, 73-80 (1994).
20 Coyle, A. J. *et al.* Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* **13**, 54-59, doi:10.1165/ajrcmb.13.1.7598937 (1995).
21 Brusselle, G., Kips, J., Joos, G., Bluethmann, H. & Pauwels, R. Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am J Respir Cell Mol Biol* **12**, 254-259, doi:10.1165/ajrcmb.12.3.7873190 (1995).
22 Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).
23 Grunig, G. *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261-2263 (1998).
24 Cianchetti, S. *et al.* Are sputum eosinophil cationic protein and eosinophils differently associated with clinical and functional findings of asthma? *Clin Exp Allergy* **44**, 673-680, doi:10.1111/cea.12236 (2014).
25 Duncan, C. J., Lawrie, A., Blaylock, M. G., Douglas, J. G. & Walsh, G. M. Reduced eosinophil apoptosis in induced sputum correlates with asthma severity. *Eur Respir J* **22**, 484-490 (2003).
26 Denlinger, L. C. *et al.* Inflammatory and Comorbid Features of Patients with Severe Asthma and Frequent Exacerbations. *Am J Respir Crit Care Med* **195**, 302-313, doi:10.1164/rccm.201602-0419OC (2017).
27 . (!!! INVALID CITATION !!! 2,4).
28 Mesnil, C. *et al.* Lung-resident eosinophils represent a distinct regulatory eosinophil subset. *J Clin Invest* **126**, 3279-3295, doi:10.1172/JCI85664 (2016).
29 Tomaki, M. *et al.* Eosinophilopoiesis in a murine model of allergic airway eosinophilia: involvement of bone marrow IL-5 and IL-5 receptor alpha. *J Immunol* **165**, 4040-4050 (2000).
30 Inman, M. D., Ellis, R., Wattie, J., Denburg, J. A. & O'Byrne, P. M. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am J Respir Cell Mol Biol* **21**, 473-479, doi:10.1165/ajrcmb.21.4.3622 (1999).
31 Shen, H. *et al.* The effects of intranasal budesonide on allergen-induced production of interleukin-5 and eotaxin, airways, blood, and bone marrow eosinophilia, and eosinophil progenitor expansion in sensitized mice. *Am J Respir Crit Care Med* **166**, 146-153, doi:10.1164/rccm.2008161 (2002).
32 Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B. & Robinson, D. S. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med* **167**, 199-204, doi:10.1164/rccm.200208-789OC (2003).
33 Kelly, E. A. *et al.* Mepolizumab Attenuates Airway Eosinophil Numbers, but Not Their Functional Phenotype, in Asthma. *Am J Respir Crit Care Med* **196**, 1385-1395, doi:10.1164/rccm.201611-2234OC (2017).

34 Jacobsen, E. A. *et al.* Eosinophil activities modulate the immune/inflammatory character of allergic respiratory responses in mice. *Allergy* **69**, 315-327, doi:10.1111/all.12321 (2014).

35 Lee, J. J. *et al.* Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* **305**, 1773-1776, doi:10.1126/science.1099472 (2004).

36 Shen, H. H. *et al.* A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *J Immunol* **170**, 3296-3305 (2003).

37 Lee, J. J. *et al.* Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* **185**, 2143-2156 (1997).

38 Aalbers, R. *et al.* Dynamics of eosinophil infiltration in the bronchial mucosa before and after the late asthmatic reaction. *Eur Respir J* **6**, 840-847 (1993).

39 Davoine, F. & Lacy, P. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Front Immunol* **5**, 570, doi:10.3389/fimmu.2014.00570 (2014).

40 Spencer, L. A. *et al.* Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J Leukoc Biol* **85**, 117-123, doi:10.1189/jlb.0108058 (2009).

41 Jacobsen, E. A. *et al.* Lung Pathologies in a Chronic Inflammation Mouse Model Are Independent of Eosinophil Degranulation. *Am J Respir Crit Care Med* **195**, 1321-1332, doi:10.1164/rccm.201606-1129OC (2017).

42 Evans, C. M., Fryer, A. D., Jacoby, D. B., Gleich, G. J. & Costello, R. W. Pretreatment with antibody to eosinophil major basic protein prevents hyperresponsiveness by protecting neuronal M2 muscarinic receptors in antigen-challenged guinea pigs. *J Clin Invest* **100**, 2254-2262, doi:10.1172/JCI119763 (1997).

43 Denzler, K. L. *et al.* Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J Immunol* **165**, 5509-5517 (2000).

44 Doyle, A. D. *et al.* Homologous recombination into the eosinophil peroxidase locus generates a strain of mice expressing Cre recombinase exclusively in eosinophils. *J Leukoc Biol* **94**, 17-24, doi:10.1189/jlb.0213089 (2013).

45 Takayama, G. *et al.* Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *J Allergy Clin Immunol* **118**, 98-104, doi:10.1016/j.jaci.2006.02.046 (2006).

46 Suzaki, I. *et al.* Inhibition of IL-13-induced periostin in airway epithelium attenuates cellular protein expression of MUC5AC. *Respirology* **22**, 93-100, doi:10.1111/resp.12873 (2017).

47 Simpson, J. L. *et al.* Periostin levels and eosinophilic inflammation in poorly-controlled asthma. *BMC Pulm Med* **16**, 67, doi:10.1186/s12890-016-0230-4 (2016).

48 Jia, G. *et al.* Periostin is a systemic biomarker of eosinophilic airway inflammation in asthmatic patients. *J Allergy Clin Immunol* **130**, 647-654 e610, doi:10.1016/j.jaci.2012.06.025 (2012).

49 Hanania, N. A. *et al.* Omalizumab in severe allergic asthma inadequately controlled with standard therapy: a randomized trial. *Ann Intern Med* **154**, 573-582, doi:10.7326/0003-4819-154-9-201105030-00002 (2011).

50 Busse, W. W. *et al.* Randomized trial of omalizumab (anti-IgE) for asthma in inner-city children. *N Engl J Med* **364**, 1005-1015, doi:10.1056/NEJMoa1009705 (2011).

51 Garcia, G. *et al.* A proof-of-concept, randomized, controlled trial of omalizumab in patients with severe, difficult-to-control, nonatopic asthma. *Chest* **144**, 411-419, doi:10.1378/chest.12-1961 (2013).
52 Nair, P. *et al.* Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* **360**, 985-993, doi:10.1056/NEJMoa0805435 (2009).
53 Bel, E. H. *et al.* Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma. *N Engl J Med* **371**, 1189-1197, doi:10.1056/NEJMoa1403291 (2014).
54 Ortega, H. G. *et al.* Mepolizumab treatment in patients with severe eosinophilic asthma. *N Engl J Med* **371**, 1198-1207, doi:10.1056/NEJMoa1403290 (2014).
55 Pavord, I. D. *et al.* Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *Lancet* **380**, 651-659, doi:10.1016/S0140-6736(12)60988-X (2012).
56 Ortega, H. G. *et al.* Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies. *Lancet Respir Med* **4**, 549-556, doi:10.1016/S2213-2600(16)30031-5 (2016).
57 Castro, M. *et al.* Reslizumab for poorly controlled, eosinophilic asthma: a randomized, placebo-controlled study. *Am J Respir Crit Care Med* **184**, 1125-1132, doi:10.1164/rccm.201103-0396OC (2011).
58 Castro, M. *et al.* Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med* **3**, 355-366, doi:10.1016/S2213-2600(15)00042-9 (2015).
59 Corren, J., Weinstein, S., Janka, L., Zangrilli, J. & Garin, M. Phase 3 Study of Reslizumab in Patients With Poorly Controlled Asthma: Effects Across a Broad Range of Eosinophil Counts. *Chest* **150**, 799-810, doi:10.1016/j.chest.2016.03.018 (2016).
60 Laviolette, M. *et al.* Effects of benralizumab on airway eosinophils in asthmatic patients with sputum eosinophilia. *J Allergy Clin Immunol* **132**, 1086-1096 e1085, doi:10.1016/j.jaci.2013.05.020 (2013).
61 Bleecker, E. R. *et al.* Efficacy and safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting beta2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial. *Lancet* **388**, 2115-2127, doi:10.1016/S0140-6736(16)31324-1 (2016).
62 FitzGerald, J. M. *et al.* Benralizumab, an anti-interleukin-5 receptor alpha monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* **388**, 2128-2141, doi:10.1016/S0140-6736(16)31322-8 (2016).
63 Nair, P. *et al.* Oral Glucocorticoid-Sparing Effect of Benralizumab in Severe Asthma. *N Engl J Med* **376**, 2448-2458, doi:10.1056/NEJMoa1703501 (2017).
64 O'Byrne, P. M. *et al.* Severe exacerbations and decline in lung function in asthma. *Am J Respir Crit Care Med* **179**, 19-24, doi:10.1164/rccm.200807-1126OC (2009).
65 Corren, J. *et al.* Lebrikizumab treatment in adults with asthma. *N Engl J Med* **365**, 1088-1098, doi:10.1056/NEJMoa1106469 (2011).
66 Hanania, N. A. *et al.* Lebrikizumab in moderate-to-severe asthma: pooled data from two randomised placebo-controlled studies. *Thorax* **70**, 748-756, doi:10.1136/thoraxjnl-2014-206719 (2015).
67 Hanania, N. A. *et al.* Efficacy and safety of lebrikizumab in patients with uncontrolled asthma (LAVOLTA I and LAVOLTA II): replicate, phase 3,

randomised, double-blind, placebo-controlled trials. *Lancet Respir Med* **4**, 781-796, doi:10.1016/S2213-2600(16)30265-X (2016).

68 Wenzel, S. *et al.* Dupilumab efficacy and safety in adults with uncontrolled persistent asthma despite use of medium-to-high-dose inhaled corticosteroids plus a long-acting beta2 agonist: a randomised double-blind placebo-controlled pivotal phase 2b dose-ranging trial. *Lancet* **388**, 31-44, doi:10.1016/S0140-6736(16)30307-5 (2016).

69 Stern, D. A., Morgan, W. J., Halonen, M., Wright, A. L. & Martinez, F. D. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. *Lancet* **372**, 1058-1064, doi:10.1016/S0140-6736(08)61447-6 (2008).

70 Fleming, L. *et al.* The burden of severe asthma in childhood and adolescence: results from the paediatric U-BIOPRED cohorts. *Eur Respir J* **46**, 1322-1333, doi:10.1183/13993003.00780-2015 (2015).

71 McGeachie, M. J. *et al.* Patterns of Growth and Decline in Lung Function in Persistent Childhood Asthma. *N Engl J Med* **374**, 1842-1852, doi:10.1056/NEJMoa1513737 (2016).

72 Stern, D. A., Morgan, W. J., Wright, A. L., Guerra, S. & Martinez, F. D. Poor airway function in early infancy and lung function by age 22 years: a non-selective longitudinal cohort study. *Lancet* **370**, 758-764, doi:10.1016/S0140-6736(07)61379-8 (2007).

73 Haland, G. *et al.* Reduced lung function at birth and the risk of asthma at 10 years of age. *N Engl J Med* **355**, 1682-1689, doi:10.1056/NEJMoa052885 (2006).

74 Owens, L., Laing, I. A., Zhang, G. & Le Souef, P. N. Infant lung function predicts asthma persistence and remission in young adults. *Respirology* **22**, 289-294, doi:10.1111/resp.12901 (2017).

75 Tai, A. *et al.* Outcomes of childhood asthma to the age of 50 years. *J Allergy Clin Immunol* **133**, 1572-1578 e1573, doi:10.1016/j.jaci.2013.12.1033 (2014).

76 Martinez, F. D. *et al.* Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med* **332**, 133-138, doi:10.1056/NEJM199501193320301 (1995).

77 Martin, A. J., McLennan, L. A., Landau, L. I. & Phelan, P. D. The natural history of childhood asthma to adult life. *Br Med J* **280**, 1397-1400 (1980).

78 Illi, S. *et al.* Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. *Lancet* **368**, 763-770, doi:10.1016/S0140-6736(06)69286-6 (2006).

79 Arshad, S. H., Stevens, M. & Hide, D. W. The effect of genetic and environmental factors on the prevalence of allergic disorders at the age of two years. *Clin Exp Allergy* **23**, 504-511 (1993).

80 Feldman, A. S., He, Y., Moore, M. L., Hershenson, M. B. & Hartert, T. V. Toward primary prevention of asthma. Reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthma. *Am J Respir Crit Care Med* **191**, 34-44, doi:10.1164/rccm.201405-0901PP (2015).

81 Sonnenschein-van der Voort, A. M. *et al.* Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. *J Allergy Clin Immunol* **133**, 1317-1329, doi:10.1016/j.jaci.2013.12.1082 (2014).

82 Sears, M. R. *et al.* A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. *N Engl J Med* **349**, 1414-1422, doi:10.1056/NEJMoa022363 (2003).

83 Kelly, Y. J. *et al.* Maternal asthma, premature birth, and the risk of respiratory morbidity in schoolchildren in Merseyside. *Thorax* **50**, 525-530 (1995).

84 Paaso, E. M., Jaakkola, M. S., Rantala, A. K., Hugg, T. T. & Jaakkola, J. J. Allergic diseases and asthma in the family predict the persistence and onset-age of asthma: a prospective cohort study. *Respir Res* **15**, 152, doi:10.1186/s12931-014-0152-8 (2014).

85 Lim, R. H., Kobzik, L. & Dahl, M. Risk for asthma in offspring of asthmatic mothers versus fathers: a meta-analysis. *PLoS One* **5**, e10134, doi:10.1371/journal.pone.0010134 (2010).

86 Martel, M. J. *et al.* Control and severity of asthma during pregnancy are associated with asthma incidence in offspring: two-stage case-control study. *Eur Respir J* **34**, 579-587, doi:10.1183/09031936.00074608 (2009).

87 Liu, X. *et al.* Maternal asthma severity and control during pregnancy and risk of offspring asthma. *J Allergy Clin Immunol* **141**, 886-892 e883, doi:10.1016/j.jaci.2017.05.016 (2018).

88 Mattes, J., Murphy, V. E., Powell, H. & Gibson, P. G. Prenatal origins of bronchiolitis: protective effect of optimised asthma management during pregnancy. *Thorax* **69**, 383-384, doi:10.1136/thoraxjnl-2013-203388 (2014).

89 Morten, M. *et al.* Managing Asthma in Pregnancy (MAP) trial: FeNO levels and childhood asthma. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2018.02.039 (2018).

90 Fisher, J. T., Brundage, K. L., Waldron, M. A. & Connelly, B. J. Vagal cholinergic innervation of the airways in newborn cat and dog. *J Appl Physiol (1985)* **69**, 1525-1531, doi:10.1152/jappl.1990.69.4.1525 (1990).

91 Lowe, L. *et al.* Specific airway resistance in 3-year-old children: a prospective cohort study. *Lancet* **359**, 1904-1908, doi:10.1016/S0140-6736(02)08781-0 (2002).

92 Young, S. *et al.* The influence of a family history of asthma and parental smoking on airway responsiveness in early infancy. *N Engl J Med* **324**, 1168-1173, doi:10.1056/NEJM199104253241704 (1991).

93 Bisgaard, H., Jensen, S. M. & Bonnelykke, K. Interaction between asthma and lung function growth in early life. *Am J Respir Crit Care Med* **185**, 1183-1189, doi:10.1164/rccm.201110-1922OC (2012).

94 Palmer, L. J. *et al.* Airway responsiveness in early infancy predicts asthma, lung function, and respiratory symptoms by school age. *Am J Respir Crit Care Med* **163**, 37-42, doi:10.1164/ajrccm.163.1.2005013 (2001).

95 Sears, M. R. *et al.* Relation between airway responsiveness and serum IgE in children with asthma and in apparently normal children. *N Engl J Med* **325**, 1067-1071, doi:10.1056/NEJM199110103251504 (1991).

96 Warner, J. A., Jones, A. C., Miles, E. A., Colwell, B. M. & Warner, J. O. Prenatal origins of asthma and allergy. *Ciba Found Symp* **206**, 220-228; discussion 228-232 (1997).

97 Devereux, G., Barker, R. N. & Seaton, A. Antenatal determinants of neonatal immune responses to allergens. *Clin Exp Allergy* **32**, 43-50 (2002).

98 Celedon, J. C. *et al.* Exposure to cat allergen, maternal history of asthma, and wheezing in first 5 years of life. *Lancet* **360**, 781-782, doi:10.1016/S0140-6736(02)09906-3 (2002).

99 Berankova, K., Uhlik, J., Honkova, L. & Pohunek, P. Structural changes in the bronchial mucosa of young children at risk of developing asthma. *Pediatr Allergy Immunol* **25**, 136-142, doi:10.1111/pai.12119 (2014).

100 Lopez-Guisa, J. M. *et al.* Airway epithelial cells from asthmatic children differentially express proremodeling factors. *J Allergy Clin Immunol* **129**, 990-997 e996, doi:10.1016/j.jaci.2011.11.035 (2012).
101 Gagliardo, R. *et al.* Non-invasive markers of airway inflammation and remodeling in childhood asthma. *Pediatr Allergy Immunol* **20**, 780-790, doi:10.1111/j.1399-3038.2009.00945.x (2009).
102 Barton, S. J. *et al.* DNA methylation of Th2 lineage determination genes at birth is associated with allergic outcomes in childhood. *Clin Exp Allergy* **47**, 1599-1608, doi:10.1111/cea.12988 (2017).
103 DeVries, A. *et al.* Epigenome-wide analysis links SMAD3 methylation at birth to asthma in children of asthmatic mothers. *J Allergy Clin Immunol* **140**, 534-542, doi:10.1016/j.jaci.2016.10.041 (2017).
104 Yang, I. V. *et al.* The nasal methylome and childhood atopic asthma. *J Allergy Clin Immunol* **139**, 1478-1488, doi:10.1016/j.jaci.2016.07.036 (2017).
105 Xu, C. J. *et al.* DNA methylation in childhood asthma: an epigenome-wide meta-analysis. *Lancet Respir Med*, doi:10.1016/S2213-2600(18)30052-3 (2018).
106 Forno, E. *et al.* A Multiomics Approach to Identify Genes Associated with Childhood Asthma Risk and Morbidity. *Am J Respir Cell Mol Biol* **57**, 439-447, doi:10.1165/rcmb.2017-0002OC (2017).
107 Arathimos, R. *et al.* Epigenome-wide association study of asthma and wheeze in childhood and adolescence. *Clin Epigenetics* **9**, 112, doi:10.1186/s13148-017-0414-7 (2017).
108 Yang, I. V. *et al.* DNA methylation and childhood asthma in the inner city. *J Allergy Clin Immunol* **136**, 69-80, doi:10.1016/j.jaci.2015.01.025 (2015).
109 Michel, S. *et al.* Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. *Allergy* **68**, 355-364, doi:10.1111/all.12097 (2013).
110 Brand, S. *et al.* DNA methylation of TH1/TH2 cytokine genes affects sensitization and progress of experimental asthma. *J Allergy Clin Immunol* **129**, 1602-1610 e1606, doi:10.1016/j.jaci.2011.12.963 (2012).
111 Cheng, R. Y. *et al.* Alterations of the lung methylome in allergic airway hyper-responsiveness. *Environ Mol Mutagen* **55**, 244-255, doi:10.1002/em.21851 (2014).
112 Shang, Y. *et al.* Epigenetic alterations by DNA methylation in house dust mite-induced airway hyperresponsiveness. *Am J Respir Cell Mol Biol* **49**, 279-287, doi:10.1165/rcmb.2012-0403OC (2013).
113 Nicodemus-Johnson, J. *et al.* Genome-Wide Methylation Study Identifies an IL-13-induced Epigenetic Signature in Asthmatic Airways. *Am J Respir Crit Care Med* **193**, 376-385, doi:10.1164/rccm.201506-1243OC (2016).
114 Nicodemus-Johnson, J. *et al.* DNA methylation in lung cells is associated with asthma endotypes and genetic risk. *JCI Insight* **1**, e90151, doi:10.1172/jci.insight.90151 (2016).
115 Gunawardhana, L. P. *et al.* Differential DNA methylation profiles of infants exposed to maternal asthma during pregnancy. *Pediatr Pulmonol* **49**, 852-862, doi:10.1002/ppul.22930 (2014).
116 Niedzwiecki, M. *et al.* Prenatal exposure to allergen, DNA methylation, and allergy in grandoffspring mice. *Allergy* **67**, 904-910, doi:10.1111/j.1398-9995.2012.02841.x (2012).
117 Braback, L. *et al.* Childhood asthma and smoking exposures before conception - a three-generational cohort study. *Pediatr Allergy Immunol*, doi:10.1111/pai.12883 (2018).

118 Lodge, C. J. *et al.* Grandmaternal smoking increases asthma risk in grandchildren: A nationwide Swedish cohort. *Clin Exp Allergy* **48**, 167-174, doi:10.1111/cea.13031 (2018).

119 Singh, S. P. *et al.* Gestational Exposure to Sidestream (Secondhand) Cigarette Smoke Promotes Transgenerational Epigenetic Transmission of Exacerbated Allergic Asthma and Bronchopulmonary Dysplasia. *J Immunol* **198**, 3815-3822, doi:10.4049/jimmunol.1700014 (2017).

120 Hamada, K. *et al.* Allergen-independent maternal transmission of asthma susceptibility. *J Immunol* **170**, 1683-1689 (2003).

121 Chen, J. C. *et al.* Fetal Phagocytes Take up Allergens to Initiate T-Helper Cell Type 2 Immunity and Facilitate Allergic Airway Responses. *Am J Respir Crit Care Med* **194**, 934-947, doi:10.1164/rccm.201508-1703OC (2016).

122 Bahrainwala, A., Hassan, S., Long, M. & Kaplan, J. Cord blood house dust mite allergen in newborns: relationship to maternal blood levels of allergen and allergen specific IgG and IgE. *Ann Allergy Asthma Immunol* **95**, 480-483, doi:10.1016/S1081-1206(10)61175-1 (2005).

123 Macchiaverni, P. *et al.* Mother to child transfer of IgG and IgA antibodies against Dermatophagoides pteronyssinus. *Scand J Immunol* **74**, 619-627, doi:10.1111/j.1365-3083.2011.02615.x (2011).

124 Rowe, J. *et al.* Prenatal versus postnatal sensitization to environmental allergens in a high-risk birth cohort. *J Allergy Clin Immunol* **119**, 1164-1173, doi:10.1016/j.jaci.2007.02.016 (2007).

125 Richgels, P. K., Yamani, A., Chougnet, C. A. & Lewkowich, I. P. Maternal house dust mite exposure during pregnancy enhances severity of house dust mite-induced asthma in murine offspring. *J Allergy Clin Immunol* **140**, 1404-1415 e1409, doi:10.1016/j.jaci.2016.12.972 (2017).

126 Lima, C. *et al.* Modulation of the induction of lung and airway allergy in the offspring of IFN-gamma-treated mother mice. *J Immunol* **175**, 3554-3559 (2005).

127 Blumer, N., Herz, U., Wegmann, M. & Renz, H. Prenatal lipopolysaccharide-exposure prevents allergic sensitization and airway inflammation, but not airway responsiveness in a murine model of experimental asthma. *Clin Exp Allergy* **35**, 397-402, doi:10.1111/j.1365-2222.2005.02184.x (2005).

128 Kim, J. H., Kim, K. H., Woo, H. Y. & Shim, J. Y. Maternal cytokine production during pregnancy and the development of childhood wheezing and allergic disease in offspring three years of age. *J Asthma* **45**, 948-952, doi:10.1080/02770900802419676 (2008).

129 Soto-Ramirez, N. *et al.* Maternal immune markers in serum during gestation and in breast milk and the risk of asthma-like symptoms at ages 6 and 12 months: a longitudinal study. *Allergy Asthma Clin Immunol* **8**, 11, doi:10.1186/1710-1492-8-11 (2012).

130 Aaltonen, R., Heikkinen, T., Hakala, K., Laine, K. & Alanen, A. Transfer of proinflammatory cytokines across term placenta. *Obstet Gynecol* **106**, 802-807, doi:10.1097/01.AOG.0000178750.84837.ed (2005).

131 Zaretsky, M. V., Alexander, J. M., Byrd, W. & Bawdon, R. E. Transfer of inflammatory cytokines across the placenta. *Obstet Gynecol* **103**, 546-550, doi:10.1097/01.AOG.0000114980.40445.83 (2004).

132 Reisenberger, K. *et al.* The transfer of interleukin-8 across the human placenta perfused in vitro. *Obstet Gynecol* **87**, 613-616, doi:10.1016/0029-7844(95)00473-4 (1996).

133 Dilworth, M. R. & Sibley, C. P. Review: Transport across the placenta of mice and women. *Placenta* **34 Suppl**, S34-39, doi:10.1016/j.placenta.2012.10.011 (2013).
134 Furukawa, S., Kuroda, Y. & Sugiyama, A. A comparison of the histological structure of the placenta in experimental animals. *J Toxicol Pathol* **27**, 11-18, doi:10.1293/tox.2013-0060 (2014).
135 Lim, R. H. & Kobzik, L. Transplacental passage of interleukins 4 and 13? *PLoS One* **4**, e4660, doi:10.1371/journal.pone.0004660 (2009).
136 Topping, V. *et al.* Interleukin-33 in the human placenta. *J Matern Fetal Neonatal Med* **26**, 327-338, doi:10.3109/14767058.2012.735724 (2013).
137 Keelan, J. A. *et al.* Cytokine abundance in placental tissues: evidence of inflammatory activation in gestational membranes with term and preterm parturition. *Am J Obstet Gynecol* **181**, 1530-1536 (1999).
138 Steinborn, A., von Gall, C., Hildenbrand, R., Stutte, H. J. & Kaufmann, M. Identification of placental cytokine-producing cells in term and preterm labor. *Obstet Gynecol* **91**, 329-335 (1998).
139 Abelius, M. S. *et al.* The placental immune milieu is characterized by a Th2- and anti-inflammatory transcription profile, regardless of maternal allergy, and associates with neonatal immunity. *Am J Reprod Immunol* **73**, 445-459, doi:10.1111/aji.12350 (2015).
140 Gayle, D. A. *et al.* Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. *Am J Physiol Regul Integr Comp Physiol* **286**, R1024-1029, doi:10.1152/ajpregu.00664.2003 (2004).
141 Scott, N. M. *et al.* Placental cytokine expression covaries with maternal asthma severity and fetal sex. *J Immunol* **182**, 1411-1420 (2009).
142 Barker, D. J. The developmental origins of chronic adult disease. *Acta Paediatr Suppl* **93**, 26-33 (2004).
143 Lawlor, D. A., Ebrahim, S. & Davey Smith, G. Association of birth weight with adult lung function: findings from the British Women's Heart and Health Study and a meta-analysis. *Thorax* **60**, 851-858, doi:10.1136/thx.2005.042408 (2005).
144 Barker, D. J. *et al.* Foetal and childhood growth and asthma in adult life. *Acta Paediatr* **102**, 732-738, doi:10.1111/apa.12257 (2013).
145 Barker, D. J. & Thornburg, K. L. Placental programming of chronic diseases, cancer and lifespan: a review. *Placenta* **34**, 841-845, doi:10.1016/j.placenta.2013.07.063 (2013).
146 Murphy, V. E. *et al.* Maternal asthma is associated with reduced female fetal growth. *Am J Respir Crit Care Med* **168**, 1317-1323, doi:10.1164/rccm.200303-374OC (2003).
147 Breton, M. C. *et al.* Risk of perinatal mortality associated with asthma during pregnancy. *Thorax* **64**, 101-106, doi:10.1136/thx.2008.102970 (2009).
148 Mayhew, T. M., Jenkins, H., Todd, B. & Clifton, V. L. Maternal asthma and placental morphometry: effects of severity, treatment and fetal sex. *Placenta* **29**, 366-373, doi:10.1016/j.placenta.2008.01.011 (2008).
149 Ricco, M. M., Kummer, W., Biglari, B., Myers, A. C. & Undem, B. J. Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways. *J Physiol* **496 (Pt 2)**, 521-530 (1996).
150 Liu, J., Song, N., Guardiola, J., Roman, J. & Yu, J. Slowly Adapting Sensory Units Have More Receptors in Large Airways than in Small Airways in Rabbits. *Front Physiol* **7**, 588, doi:10.3389/fphys.2016.00588 (2016).

151 Yu, J., Wang, Y. F. & Zhang, J. W. Structure of slowly adapting pulmonary stretch receptors in the lung periphery. *J Appl Physiol (1985)* **95**, 385-393, doi:10.1152/japplphysiol.00137.2003 (2003).
152 Hunter, D. D. & Undem, B. J. Identification and substance P content of vagal afferent neurons innervating the epithelium of the guinea pig trachea. *Am J Respir Crit Care Med* **159**, 1943-1948, doi:10.1164/ajrccm.159.6.9808078 (1999).
153 De Troyer, A., Yernault, J. C. & Rodenstein, D. Effects of vagal blockade on lung mechanics in normal man. *J Appl Physiol Respir Environ Exerc Physiol* **46**, 217-226, doi:10.1152/jappl.1979.46.2.217 (1979).
154 Simonsson, B. G., Jacobs, F. M. & Nadel, J. A. Role of autonomic nervous system and the cough reflex in the increased responsiveness of airways in patients with obstructive airway disease. *J Clin Invest* **46**, 1812-1818, doi:10.1172/JCI105671 (1967).
155 Kajekar, R., Rohde, H. K. & Myers, A. C. The integrative membrane properties of human bronchial parasympathetic Ganglia neurons. *Am J Respir Crit Care Med* **164**, 1927-1932, doi:10.1164/ajrccm.164.10.2106073 (2001).
156 Coburn, R. F. & Kalia, M. P. Morphological features of spiking and nonspiking cells in the paratracheal ganglion of the ferret. *J Comp Neurol* **254**, 341-351, doi:10.1002/cne.902540307 (1986).
157 Myers, A. C. & Undem, B. J. Analysis of preganglionic nerve evoked cholinergic contractions of the guinea pig bronchus. *J Auton Nerv Syst* **35**, 175-184 (1991).
158 Fisher, J. T., Vincent, S. G., Gomeza, J., Yamada, M. & Wess, J. Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M2 or M3 muscarinic acetylcholine receptors. *FASEB J* **18**, 711-713, doi:10.1096/fj.03-0648fje (2004).
159 Faulkner, D., Fryer, A. D. & Maclagan, J. Postganglionic muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* **88**, 181-187 (1986).
160 Fryer, A. D. & Maclagan, J. Muscarinic inhibitory receptors in pulmonary parasympathetic nerves in the guinea-pig. *Br J Pharmacol* **83**, 973-978 (1984).
161 Canning, B. J., Undem, B. J., Karakousis, P. C. & Dey, R. D. Effects of organotypic culture on parasympathetic innervation of guinea pig trachealis. *Am J Physiol* **271**, L698-706, doi:10.1152/ajplung.1996.271.5.L698 (1996).
162 Balentova, S., Conwell, S. & Myers, A. C. Neurotransmitters in parasympathetic ganglionic neurons and nerves in mouse lower airway smooth muscle. *Respir Physiol Neurobiol* **189**, 195-202, doi:10.1016/j.resp.2013.07.006 (2013).
163 Dinh, Q. T. *et al.* Expression of substance P and nitric oxide synthase in vagal sensory neurons innervating the mouse airways. *Regul Pept* **126**, 189-194, doi:10.1016/j.regpep.2004.09.006 (2005).
164 Dey, R. D., Mayer, B. & Said, S. I. Colocalization of vasoactive intestinal peptide and nitric oxide synthase in neurons of the ferret trachea. *Neuroscience* **54**, 839-843 (1993).
165 Canning, B. J. & Undem, B. J. Evidence that antidromically stimulated vagal afferents activate inhibitory neurones innervating guinea-pig trachealis. *J Physiol* **480 (Pt 3)**, 613-625 (1994).
166 Langley, J. N. Antidromic action: Part II. Stimulation of the peripheral nerves of the cat's hind foot. *J Physiol* **58**, 49-69 (1923).
167 Brodin, E., Gazelius, B., Olgart, L. & Nilsson, G. Tissue concentration and release of substance P-like immunoreactivity in the dental pulp. *Acta Physiol Scand* **111**, 141-149, doi:10.1111/j.1748-1716.1981.tb06717.x (1981).

168 El-Bermani, A. W. Pulmonary noradrenergic innervation of rat and monkey: a comparative study. *Thorax* **33**, 167-174 (1978).
169 Doidge, J. M. & Satchell, D. G. Adrenergic and non-adrenergic inhibitory nerves in mammalian airways. *J Auton Nerv Syst* **5**, 83-99 (1982).
170 Russell, J. A. Noradrenergic inhibitory innervation of canine airways. *J Appl Physiol Respir Environ Exerc Physiol* **48**, 16-22, doi:10.1152/jappl.1980.48.1.16 (1980).
171 Matsumoto, N. *et al.* Effective sites by sympathetic beta-adrenergic and vagal nonadrenergic inhibitory stimulation in constricted airways. *Am Rev Respir Dis* **132**, 1113-1117, doi:10.1164/arrd.1985.132.5.1113 (1985).
172 Harris, L. Comparison of cardiorespiratory effects of terbutaline and salbutamol aerosols in patients with reversible airways obstruction. *Thorax* **28**, 592-595 (1973).
173 Wagner, E. M. & Jacoby, D. B. Methacholine causes reflex bronchoconstriction. *J Appl Physiol (1985)* **86**, 294-297, doi:10.1152/jappl.1999.86.1.294 (1999).
174 McAlexander, M. A., Gavett, S. H., Kollarik, M. & Undem, B. J. Vagotomy reverses established allergen-induced airway hyperreactivity to methacholine in the mouse. *Respir Physiol Neurobiol* **212-214**, 20-24, doi:10.1016/j.resp.2015.03.007 (2015).
175 Mills, J. E. & Widdicombe, J. G. Role of the vagus nerves in anaphylaxis and histamine-induced bronchoconstrictions in guinea-pigs. *Br J Pharmacol* **39**, 724-731 (1970).
176 Costello, R. W. *et al.* Antigen-induced hyperreactivity to histamine: role of the vagus nerves and eosinophils. *Am J Physiol* **276**, L709-714 (1999).
177 Roberts, A. M. *et al.* Reflex tracheal contraction induced by stimulation of bronchial C-fibers in dogs. *J Appl Physiol Respir Environ Exerc Physiol* **51**, 485-493, doi:10.1152/jappl.1981.51.2.485 (1981).
178 Fuller, R. W., Dixon, C. M. & Barnes, P. J. Bronchoconstrictor response to inhaled capsaicin in humans. *J Appl Physiol (1985)* **58**, 1080-1084, doi:10.1152/jappl.1985.58.4.1080 (1985).
179 Potenzieri, C., Meeker, S. & Undem, B. J. Activation of mouse bronchopulmonary C-fibres by serotonin and allergen-ovalbumin challenge. *J Physiol* **590**, 5449-5459, doi:10.1113/jphysiol.2012.237115 (2012).
180 Gold, W. M., Kessler, G. F. & Yu, D. Y. Role of vagus nerves in experimental asthma in allergic dogs. *J Appl Physiol* **33**, 719-725, doi:10.1152/jappl.1972.33.6.719 (1972).
181 Sakae, R. S. *et al.* Neonatal capsaicin treatment decreases airway and pulmonary tissue responsiveness to methacholine. *Am J Physiol* **266**, L23-29, doi:10.1152/ajplung.1994.266.1.L23 (1994).
182 Peters, S. P. *et al.* Tiotropium bromide step-up therapy for adults with uncontrolled asthma. *N Engl J Med* **363**, 1715-1726, doi:10.1056/NEJMoa1008770 (2010).
183 Kerstjens, H. A. *et al.* Tiotropium in asthma poorly controlled with standard combination therapy. *N Engl J Med* **367**, 1198-1207, doi:10.1056/NEJMoa1208606 (2012).
184 Szefler, S. J. *et al.* A phase III randomized controlled trial of tiotropium add-on therapy in children with severe symptomatic asthma. *J Allergy Clin Immunol* **140**, 1277-1287, doi:10.1016/j.jaci.2017.01.014 (2017).
185 Hamelmann, E. *et al.* A randomised controlled trial of tiotropium in adolescents with severe symptomatic asthma. *Eur Respir J* **49**, doi:10.1183/13993003.01100-2016 (2017).

186 Trankner, D., Hahne, N., Sugino, K., Hoon, M. A. & Zuker, C. Population of sensory neurons essential for asthmatic hyperreactivity of inflamed airways. *Proc Natl Acad Sci U S A* **111**, 11515-11520, doi:10.1073/pnas.1411032111 (2014).
187 Zhang, G., Lin, R. L., Wiggers, M. E. & Lee, L. Y. Sensitizing effects of chronic exposure and acute inhalation of ovalbumin aerosol on pulmonary C fibers in rats. *J Appl Physiol (1985)* **105**, 128-138, doi:10.1152/japplphysiol.01367.2007 (2008).
188 Riccio, M. M., Myers, A. C. & Undem, B. J. Immunomodulation of afferent neurons in guinea-pig isolated airway. *J Physiol* **491 (Pt 2)**, 499-509 (1996).
189 Hooper, J. S. *et al.* Characterization of cardiovascular reflexes evoked by airway stimulation with allylisothiocyanate, capsaicin, and ATP in Sprague-Dawley rats. *J Appl Physiol (1985)* **120**, 580-591, doi:10.1152/japplphysiol.00944.2015 (2016).
190 McQueen, D. S. *et al.* Activation of P2X receptors for adenosine triphosphate evokes cardiorespiratory reflexes in anaesthetized rats. *J Physiol* **507 (Pt 3)**, 843-855 (1998).
191 Taylor-Clark, T. E., Nassenstein, C. & Undem, B. J. Leukotriene D4 increases the excitability of capsaicin-sensitive nasal sensory nerves to electrical and chemical stimuli. *Br J Pharmacol* **154**, 1359-1368, doi:10.1038/bjp.2008.196 (2008).
192 Maher, S. A. *et al.* Prostaglandin D2 and the role of the DP1, DP2 and TP receptors in the control of airway reflex events. *Eur Respir J* **45**, 1108-1118, doi:10.1183/09031936.00061614 (2015).
193 Inoue, H., Koto, H., Takata, S., Aizawa, H. & Ikeda, T. Excitatory role of axon reflex in bradykinin-induced contraction of guinea pig tracheal smooth muscle. *Am Rev Respir Dis* **146**, 1548-1552, doi:10.1164/ajrccm/146.6.1548 (1992).
194 Reznikov, L. R. *et al.* Acid-Sensing Ion Channel 1a Contributes to Airway Hyperreactivity in Mice. *PLoS One* **11**, e0166089, doi:10.1371/journal.pone.0166089 (2016).
195 Yermolaieva, O., Leonard, A. S., Schnizler, M. K., Abboud, F. M. & Welsh, M. J. Extracellular acidosis increases neuronal cell calcium by activating acid-sensing ion channel 1a. *Proc Natl Acad Sci U S A* **101**, 6752-6757, doi:10.1073/pnas.0308636100 (2004).
196 Gu, Q. & Lee, L. Y. Regulation of acid signaling in rat pulmonary sensory neurons by protease-activated receptor-2. *Am J Physiol Lung Cell Mol Physiol* **298**, L454-461, doi:10.1152/ajplung.00381.2009 (2010).
197 Gu, Q. & Lee, L. Y. Hypersensitivity of pulmonary chemosensitive neurons induced by activation of protease-activated receptor-2 in rats. *J Physiol* **574**, 867-876, doi:10.1113/jphysiol.2006.110312 (2006).
198 Kwong, K., Nassenstein, C., de Garavilla, L., Meeker, S. & Undem, B. J. Thrombin and trypsin directly activate vagal C-fibres in mouse lung via protease-activated receptor-1. *J Physiol* **588**, 1171-1177, doi:10.1113/jphysiol.2009.181669 (2010).
199 Oetjen, L. K. *et al.* Sensory Neurons Co-opt Classical Immune Signaling Pathways to Mediate Chronic Itch. *Cell* **171**, 217-228 e213, doi:10.1016/j.cell.2017.08.006 (2017).
200 Yu, J., Lin, S., Zhang, J., Otmishi, P. & Guardiola, J. J. Airway nociceptors activated by pro-inflammatory cytokines. *Respir Physiol Neurobiol* **156**, 116-119, doi:10.1016/j.resp.2006.11.005 (2007).
201 Makwana, R., Gozzard, N., Spina, D. & Page, C. TNF-alpha-induces airway hyperresponsiveness to cholinergic stimulation in guinea pig airways. *Br J Pharmacol* **165**, 1978-1991, doi:10.1111/j.1476-5381.2011.01675.x (2012).

202 Lee, L. Y., Gu, Q. & Gleich, G. J. Effects of human eosinophil granule-derived cationic proteins on C-fiber afferents in the rat lung. *J Appl Physiol (1985)* **91**, 1318-1326, doi:10.1152/jappl.2001.91.3.1318 (2001).
203 Kwong, K. & Lee, L. Y. PGE(2) sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli. *J Appl Physiol (1985)* **93**, 1419-1428, doi:10.1152/japplphysiol.00382.2002 (2002).
204 Hsu, C. C., Lin, Y. S., Lin, R. L. & Lee, L. Y. Immediate and delayed potentiating effects of tumor necrosis factor-alpha on TRPV1 sensitivity of rat vagal pulmonary sensory neurons. *Am J Physiol Lung Cell Mol Physiol* **313**, L293-L304, doi:10.1152/ajplung.00235.2016 (2017).
205 Gu, Q., Wiggers, M. E., Gleich, G. J. & Lee, L. Y. Sensitization of isolated rat vagal pulmonary sensory neurons by eosinophil-derived cationic proteins. *Am J Physiol Lung Cell Mol Physiol* **294**, L544-552, doi:10.1152/ajplung.00271.2007 (2008).
206 Scott, G. D. *Sensory neuroplasticitiy in asthma* PhD thesis, Oregon Health & Science University, (2012).
207 Foster, E. L. *et al.* Eosinophils increase neuron branching in human and murine skin and in vitro. *PLoS One* **6**, e22029, doi:10.1371/journal.pone.0022029 (2011).
208 Drake, M. S., GD; Blum, ED; Lebold, KM; Nie, J; Lee, JJ; Fryer, AD; Costello, RW; Jacoby, DB. Airway Sensory Nerve Density is Increased by Eosinophils in Mice and in Human Eosinophilic Asthma. *under review* (2018).
209 Lee, J. J. *et al.* Eosinophil-dependent skin innervation and itching following contact toxicant exposure in mice. *J Allergy Clin Immunol* **135**, 477-487, doi:10.1016/j.jaci.2014.07.003 (2015).
210 Chang, M. M., Leeman, S. E. & Niall, H. D. Amino-acid sequence of substance P. *Nat New Biol* **232**, 86-87 (1971).
211 Nawa, H., Kotani, H. & Nakanishi, S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature* **312**, 729-734 (1984).
212 Kawaguchi, Y., Hoshimaru, M., Nawa, H. & Nakanishi, S. Sequence analysis of cloned cDNA for rat substance P precursor: existence of a third substance P precursor. *Biochem Biophys Res Commun* **139**, 1040-1046 (1986).
213 Harmar, A. J. *et al.* cDNA sequence of human beta-preprotachykinin, the common precursor to substance P and neurokinin A. *FEBS Lett* **208**, 67-72 (1986).
214 Krause, J. E., Chirgwin, J. M., Carter, M. S., Xu, Z. S. & Hershey, A. D. Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. *Proc Natl Acad Sci U S A* **84**, 881-885 (1987).
215 Schwyzer, R. Membrane-assisted molecular mechanism of neurokinin receptor subtype selection. *EMBO J* **6**, 2255-2259 (1987).
216 Buck, S. H., Burcher, E., Shults, C. W., Lovenberg, W. & O'Donohue, T. L. Novel pharmacology of substance K-binding sites: a third type of tachykinin receptor. *Science* **226**, 987-989 (1984).
217 McNeil, B. D. *et al.* Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* **519**, 237-241, doi:10.1038/nature14022 (2015).
218 Azimi, E. *et al.* Dual action of neurokinin-1 antagonists on Mas-related GPCRs. *JCI Insight* **1**, e89362, doi:10.1172/jci.insight.89362 (2016).
219 Dey, R. D., Altemus, J. B. & Michalkiewicz, M. Distribution of vasoactive intestinal peptide- and substance P-containing nerves originating from neurons of airway

ganglia in cat bronchi. *J Comp Neurol* **304**, 330-340, doi:10.1002/cne.903040213 (1991).

220 Baluk, P., Nadel, J. A. & McDonald, D. M. Substance P-immunoreactive sensory axons in the rat respiratory tract: a quantitative study of their distribution and role in neurogenic inflammation. *J Comp Neurol* **319**, 586-598, doi:10.1002/cne.903190408 (1992).

221 Canning, B. J., Reynolds, S. M., Anukwu, L. U., Kajekar, R. & Myers, A. C. Endogenous neurokinins facilitate synaptic transmission in guinea pig airway parasympathetic ganglia. *Am J Physiol Regul Integr Comp Physiol* **283**, R320-330, doi:10.1152/ajpregu.00001.2002 (2002).

222 Lundberg, J. M., Hokfelt, T., Martling, C. R., Saria, A. & Cuello, C. Substance P-immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissue Res* **235**, 251-261 (1984).

223 Kummer, W., Fischer, A., Kurkowski, R. & Heym, C. The sensory and sympathetic innervation of guinea-pig lung and trachea as studied by retrograde neuronal tracing and double-labelling immunohistochemistry. *Neuroscience* **49**, 715-737 (1992).

224 Dey, R. D., Altemus, J. B., Zervos, I. & Hoffpauir, J. Origin and colocalization of CGRP- and SP-reactive nerves in cat airway epithelium. *J Appl Physiol (1985)* **68**, 770-778, doi:10.1152/jappl.1990.68.2.770 (1990).

225 Dey, R. D., Hoffpauir, J. & Said, S. I. Co-localization of vasoactive intestinal peptide- and substance P-containing nerves in cat bronchi. *Neuroscience* **24**, 275-281 (1988).

226 Dey, R. D. *et al.* Neurochemical characterization of intrinsic neurons in ferret tracheal plexus. *Am J Respir Cell Mol Biol* **14**, 207-216, doi:10.1165/ajrcmb.14.3.8845170 (1996).

227 Scott, G. D., Blum, E. D., Fryer, A. D. & Jacoby, D. B. Tissue optical clearing, three-dimensional imaging, and computer morphometry in whole mouse lungs and human airways. *Am J Respir Cell Mol Biol* **51**, 43-55, doi:10.1165/rcmb.2013-0284OC (2014).

228 Dinh, Q. T. *et al.* Substance P expression in TRPV1 and trkA-positive dorsal root ganglion neurons innervating the mouse lung. *Respir Physiol Neurobiol* **144**, 15-24, doi:10.1016/j.resp.2004.08.001 (2004).

229 Lindsay, R. M. & Harmar, A. J. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* **337**, 362-364, doi:10.1038/337362a0 (1989).

230 Otten, U., Goedert, M., Mayer, N. & Lembeck, F. Requirement of nerve growth factor for development of substance P-containing sensory neurones. *Nature* **287**, 158-159 (1980).

231 McMahon, S. B., Lewin, G. R., Anand, P., Ghatei, M. A. & Bloom, S. R. Quantitative analysis of peptide levels and neurogenic extravasation following regeneration of afferents to appropriate and inappropriate targets. *Neuroscience* **33**, 67-73 (1989).

232 McMahon, S. B. & Gibson, S. Peptide expression is altered when afferent nerves reinnervate inappropriate tissue. *Neurosci Lett* **73**, 9-15 (1987).

233 Wu, Z. X., Satterfield, B. E., Fedan, J. S. & Dey, R. D. Interleukin-1beta-induced airway hyperresponsiveness enhances substance P in intrinsic neurons of ferret airway. *Am J Physiol Lung Cell Mol Physiol* **283**, L909-917, doi:10.1152/ajplung.00363.2001 (2002).

234 Lin, Y. T., Ro, L. S., Wang, H. L. & Chen, J. C. Up-regulation of dorsal root ganglia BDNF and trkB receptor in inflammatory pain: an in vivo and in vitro study. *J Neuroinflammation* **8**, 126, doi:10.1186/1742-2094-8-126 (2011).
235 Freidin, M. & Kessler, J. A. Cytokine regulation of substance P expression in sympathetic neurons. *Proc Natl Acad Sci U S A* **88**, 3200-3203 (1991).
236 Ding, M., Hart, R. P. & Jonakait, G. M. Tumor necrosis factor-alpha induces substance P in sympathetic ganglia through sequential induction of interleukin-1 and leukemia inhibitory factor. *J Neurobiol* **28**, 445-454, doi:10.1002/neu.480280405 (1995).
237 Schenker, C., Mroz, E. A. & Leeman, S. E. Release of substance P from isolated nerve endings. *Nature* **264**, 790-792 (1976).
238 Jessell, T. M., Iversen, L. L. & Cuello, A. C. Capsaicin-induced depletion of substance P from primary sensory neurones. *Brain Res* **152**, 183-188 (1978).
239 Baumgarten, C. R., O'Connor, A., Dokic, D., Schultz, K. D. & Kunkel, G. Substance P is generated in vivo following nasal challenge of allergic individuals with bradykinin. *Clin Exp Allergy* **27**, 1322-1327 (1997).
240 Tonnesen, P. & Schaffalitzky de Muckadell, O. B. Substance P and vasoactive intestinal peptide in serotonin-induced nasal secretions in normal subjects. *Allergy* **42**, 146-150 (1987).
241 Vedder, H. & Otten, U. Biosynthesis and release of tachykinins from rat sensory neurons in culture. *J Neurosci Res* **30**, 288-299, doi:10.1002/jnr.490300203 (1991).
242 Su, X., Camerer, E., Hamilton, J. R., Coughlin, S. R. & Matthay, M. A. Protease-activated receptor-2 activation induces acute lung inflammation by neuropeptide-dependent mechanisms. *J Immunol* **175**, 2598-2605 (2005).
243 Nakamura, Y. *et al.* Activation of transient receptor potential ankyrin 1 evokes nociception through substance P release from primary sensory neurons. *J Neurochem* **120**, 1036-1047, doi:10.1111/j.1471-4159.2011.07628.x (2012).
244 Yokosawa, H., Endo, S., Ohgaki, Y., Maeyama, J. & Ishii, S. Hydrolysis of substance P and its analogs by angiotensin-converting enzyme from rat lung. Characterization of endopeptidase activity of the enzyme. *J Biochem* **98**, 1293-1299 (1985).
245 Lotvall, J. O., Skoogh, B. E., Barnes, P. J. & Chung, K. F. Effects of aerosolised substance P on lung resistance in guinea-pigs: a comparison between inhibition of neutral endopeptidase and angiotensin-converting enzyme. *Br J Pharmacol* **100**, 69-72 (1990).
246 Stimler-Gerard, N. P. Neutral endopeptidase-like enzyme controls the contractile activity of substance P in guinea pig lung. *J Clin Invest* **79**, 1819-1825, doi:10.1172/JCI113023 (1987).
247 Shore, S. A., Stimler-Gerard, N. P., Coats, S. R. & Drazen, J. M. Substance P-induced bronchoconstriction in the guinea pig. Enhancement by inhibitors of neutral metalloendopeptidase and angiotensin-converting enzyme. *Am Rev Respir Dis* **137**, 331-336, doi:10.1164/ajrccm/137.2.331 (1988).
248 Shore, S. A. & Drazen, J. M. Enhanced airway responses to substance P after repeated challenge in guinea pigs. *J Appl Physiol (1985)* **66**, 955-961, doi:10.1152/jappl.1989.66.2.955 (1989).
249 Choi, H. S., Lesser, M., Cardozo, C. & Orlowski, M. Immunohistochemical localization of endopeptidase 24.15 in rat trachea, lung tissue, and alveolar macrophages. *Am J Respir Cell Mol Biol* **3**, 619-624, doi:10.1165/ajrcmb/3.6.619 (1990).

250 Devillier, P., Advenier, C., Drapeau, G., Marsac, J. & Regoli, D. Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *Br J Pharmacol* **94**, 675-684 (1988).
251 Wang, L., Sadoun, E., Stephens, R. E. & Ward, P. E. Metabolism of substance P and neurokinin A by human vascular endothelium and smooth muscle. *Peptides* **15**, 497-503 (1994).
252 Cossman, J., Neckers, L. M., Leonard, W. J. & Greene, W. C. Polymorphonuclear neutrophils express the common acute lymphoblastic leukemia antigen. *J Exp Med* **157**, 1064-1069 (1983).
253 Jacoby, D. B., Tamaoki, J., Borson, D. B. & Nadel, J. A. Influenza infection causes airway hyperresponsiveness by decreasing enkephalinase. *J Appl Physiol (1985)* **64**, 2653-2658, doi:10.1152/jappl.1988.64.6.2653 (1988).
254 Capaz, F. R. *et al.* Effect of active sensitization on the bronchopulmonary responses to tachykinins in the guinea pig. Modulation by peptidase inhibitors. *J Pharmacol Exp Ther* **266**, 812-819 (1993).
255 Lilly, C. M., Kobzik, L., Hall, A. E. & Drazen, J. M. Effects of chronic airway inflammation on the activity and enzymatic inactivation of neuropeptides in guinea pig lungs. *J Clin Invest* **93**, 2667-2674, doi:10.1172/JCI117280 (1994).
256 Dusser, D. J., Djokic, T. D., Borson, D. B. & Nadel, J. A. Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. *J Clin Invest* **84**, 900-906, doi:10.1172/JCI114251 (1989).
257 Murlas, C. G., Murphy, T. P. & Lang, Z. HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hypoactivity. *Am J Physiol* **258**, L361-368, doi:10.1152/ajplung.1990.258.6.L361 (1990).
258 Shore, S. A. & Drazen, J. M. Degradative enzymes modulate airway responses to intravenous neurokinins A and B. *J Appl Physiol (1985)* **67**, 2504-2511, doi:10.1152/jappl.1989.67.6.2504 (1989).
259 Da Silva, A., Dhuy, J., Waeldele, F., Bertrand, C. & Landry, Y. Endopeptidase 24.15 modulates bradykinin-induced contraction in guinea-pig trachea. *Eur J Pharmacol* **212**, 97-99 (1992).
260 Lundberg, J. M. & Saria, A. Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants. *Nature* **302**, 251-253 (1983).
261 Coles, S. J., Bhaskar, K. R., O'Sullivan, D. D., Neill, K. H. & Reid, L. M. Airway mucus: composition and regulation of its secretion by neuropeptides in vitro. *Ciba Found Symp* **109**, 40-60 (1984).
262 Shimura, S., Sasaki, T., Okayama, H., Sasaki, H. & Takishima, T. Effect of substance P on mucus secretion of isolated submucosal gland from feline trachea. *J Appl Physiol (1985)* **63**, 646-653, doi:10.1152/jappl.1987.63.2.646 (1987).
263 Choi, J. Y. *et al.* Substance P stimulates human airway submucosal gland secretion mainly via a CFTR-dependent process. *J Clin Invest* **119**, 1189-1200, doi:10.1172/JCI37284 (2009).
264 Lotvall, J. O., Lemen, R. J., Hui, K. P., Barnes, P. J. & Chung, K. F. Airflow obstruction after substance P aerosol: contribution of airway and pulmonary edema. *J Appl Physiol (1985)* **69**, 1473-1478, doi:10.1152/jappl.1990.69.4.1473 (1990).
265 Van Rensen, E. L., Hiemstra, P. S., Rabe, K. F. & Sterk, P. J. Assessment of microvascular leakage via sputum induction: the role of substance P and

neurokinin A in patients with asthma. *Am J Respir Crit Care Med* **165**, 1275-1279, doi:10.1164/rccm.2110092 (2002).
266 Khalil, Z. & Helme, R. D. Sequence of events in substance P-mediated plasma extravasation in rat skin. *Brain Res* **500**, 256-262 (1989).
267 Yano, H., Wershil, B. K., Arizono, N. & Galli, S. J. Substance P-induced augmentation of cutaneous vascular permeability and granulocyte infiltration in mice is mast cell dependent. *J Clin Invest* **84**, 1276-1286, doi:10.1172/JCI114295 (1989).
268 Heaney, L. G., Cross, L. J. & Ennis, M. Histamine release from bronchoalveolar lavage cells from asthmatic subjects after allergen challenge and relationship to the late asthmatic response. *Clin Exp Allergy* **28**, 196-204 (1998).
269 Fewtrell, C. M. *et al.* The effects of substance P on histamine and 5-hydroxytryptamine release in the rat. *J Physiol* **330**, 393-411 (1982).
270 Matsuda, H., Kawakita, K., Kiso, Y., Nakano, T. & Kitamura, Y. Substance P induces granulocyte infiltration through degranulation of mast cells. *J Immunol* **142**, 927-931 (1989).
271 Helme, R. D., Eglezos, A. & Hosking, C. S. Substance P induces chemotaxis of neutrophils in normal and capsaicin-treated rats. *Immunol Cell Biol* **65 (Pt 3)**, 267-269, doi:10.1038/icb.1987.30 (1987).
272 Ruff, M. R., Wahl, S. M. & Pert, C. B. Substance P receptor-mediated chemotaxis of human monocytes. *Peptides* **6 Suppl 2**, 107-111 (1985).
273 Kudlacz, E. M. & Knippenberg, R. W. In vitro and in vivo effects of tachykinins on immune cell function in guinea pig airways. *J Neuroimmunol* **50**, 119-125 (1994).
274 Aliakbari, J., Sreedharan, S. P., Turck, C. W. & Goetzl, E. J. Selective localization of vasoactive intestinal peptide and substance P in human eosinophils. *Biochem Biophys Res Commun* **148**, 1440-1445 (1987).
275 Lambrecht, B. N. *et al.* Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. *Eur J Immunol* **29**, 3815-3825, doi:10.1002/(SICI)1521-4141(199912)29:12<3815::AID-IMMU3815>3.0.CO;2-# (1999).
276 Pascual, D. W. & Bost, K. L. Substance P production by P388D1 macrophages: a possible autocrine function for this neuropeptide. *Immunology* **71**, 52-56 (1990).
277 Delgado, A. V., McManus, A. T. & Chambers, J. P. Production of tumor necrosis factor-alpha, interleukin 1-beta, interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. *Neuropeptides* **37**, 355-361 (2003).
278 Azzolina, A., Bongiovanni, A. & Lampiasi, N. Substance P induces TNF-alpha and IL-6 production through NF kappa B in peritoneal mast cells. *Biochim Biophys Acta* **1643**, 75-83 (2003).
279 Hartung, H. P., Wolters, K. & Toyka, K. V. Substance P: binding properties and studies on cellular responses in guinea pig macrophages. *J Immunol* **136**, 3856-3863 (1986).
280 Evans, C. M. *et al.* Substance P-induced airway hyperreactivity is mediated by neuronal M(2) receptor dysfunction. *Am J Physiol Lung Cell Mol Physiol* **279**, L477-486 (2000).
281 Ohtake, J. *et al.* Neuropeptide signaling through neurokinin-1 and neurokinin-2 receptors augments antigen presentation by human dendritic cells. *J Allergy Clin Immunol* **136**, 1690-1694, doi:10.1016/j.jaci.2015.06.050 (2015).
282 Maghni, K. *et al.* Airway smooth muscle cells express functional neurokinin-1 receptors and the nerve-derived preprotachykinin-a gene: regulation by passive

sensitization. *Am J Respir Cell Mol Biol* **28**, 103-110, doi:10.1165/rcmb.4635 (2003).
283 US, V. E. & Gaddum, J. H. An unidentified depressor substance in certain tissue extracts. *J Physiol* **72**, 74-87 (1931).
284 Honda, I., Kohrogi, H., Yamaguchi, T., Ando, M. & Araki, S. Enkephalinase inhibitor potentiates substance P- and capsaicin-induced bronchial smooth muscle contractions in humans. *Am Rev Respir Dis* **143**, 1416-1418, doi:10.1164/ajrccm/143.6.1416 (1991).
285 Saria, A. *et al.* Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *Am Rev Respir Dis* **137**, 1330-1335, doi:10.1164/ajrccm/137.6.1330 (1988).
286 Finney, M. J., Karlsson, J. A. & Persson, C. G. Effects of bronchoconstrictors and bronchodilators on a novel human small airway preparation. *Br J Pharmacol* **85**, 29-36 (1985).
287 Mauser, P. J. *et al.* Effect of tachykinins on airway function in cynomolgus monkeys. *Pulm Pharmacol Ther* **14**, 121-127, doi:10.1006/pupt.2001.0278 (2001).
288 Myers, A. C. & Undem, B. J. Functional interactions between capsaicin-sensitive and cholinergic nerves in the guinea pig bronchus. *J Pharmacol Exp Ther* **259**, 104-109 (1991).
289 Beleslin, D., Radmanovic, B. & Varagic, V. The effect of substance P on the superior cervical ganglion of the cat. *Br J Pharmacol Chemother* **15**, 10-13 (1960).
290 Watson, N., Maclagan, J. & Barnes, P. J. Endogenous tachykinins facilitate transmission through parasympathetic ganglia in guinea-pig trachea. *Br J Pharmacol* **109**, 751-759 (1993).
291 Myers, A. C. & Undem, B. J. Electrophysiological effects of tachykinins and capsaicin on guinea-pig bronchial parasympathetic ganglion neurones. *J Physiol* **470**, 665-679 (1993).
292 Undem, B. J., Myers, A. C., Barthlow, H. & Weinreich, D. Vagal innervation of guinea pig bronchial smooth muscle. *J Appl Physiol (1985)* **69**, 1336-1346, doi:10.1152/jappl.1990.69.4.1336 (1990).
293 Yau, W. M., Dorsett, J. A. & Youther, M. L. Calcium-dependent stimulation of acetylcholine release by substance P and vasoactive intestinal polypeptide. *Eur J Pharmacol* **120**, 241-243 (1986).
294 Tournoy, K. G. *et al.* Modulatory role of tachykinin NK1 receptor in cholinergic contraction of mouse trachea. *Eur Respir J* **21**, 3-10 (2003).
295 Tomaki, M. *et al.* Elevated substance P content in induced sputum from patients with asthma and patients with chronic bronchitis. *Am J Respir Crit Care Med* **151**, 613-617, doi:10.1164/ajrccm.151.3.7533601 (1995).
296 Nieber, K. *et al.* Substance P and beta-endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic asthma. *J Allergy Clin Immunol* **90**, 646-652 (1992).
297 Ollerenshaw, S. L., Jarvis, D., Sullivan, C. E. & Woolcock, A. J. Substance P immunoreactive nerves in airways from asthmatics and nonasthmatics. *Eur Respir J* **4**, 673-682 (1991).
298 Cardell, L. O., Uddman, R. & Edvinsson, L. Low plasma concentrations of VIP and elevated levels of other neuropeptides during exacerbations of asthma. *Eur Respir J* **7**, 2169-2173 (1994).

299 Ogawa, H. *et al.* Nerve growth factor derived from bronchial epithelium after chronic mite antigen exposure contributes to airway hyperresponsiveness by inducing hyperinnervation, and is inhibited by in vivo siRNA. *Clin Exp Allergy* **42**, 460-470, doi:10.1111/j.1365-2222.2011.03918.x (2012).

300 de Vries, A. *et al.* Airway hyper-responsiveness in allergic asthma in guinea-pigs is mediated by nerve growth factor via the induction of substance P: a potential role for trkA. *Clin Exp Allergy* **36**, 1192-1200, doi:10.1111/j.1365-2222.2006.02549.x (2006).

301 Dinh, Q. T. *et al.* Allergic airway inflammation induces tachykinin peptides expression in vagal sensory neurons innervating mouse airways. *Clin Exp Allergy* **35**, 820-825, doi:10.1111/j.1365-2222.2005.02264.x (2005).

302 Myers, A. C., Kajekar, R. & Undem, B. J. Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. *Am J Physiol Lung Cell Mol Physiol* **282**, L775-781, doi:10.1152/ajplung.00353.2001 (2002).

303 Chuaychoo, B., Hunter, D. D., Myers, A. C., Kollarik, M. & Undem, B. J. Allergen-induced substance P synthesis in large-diameter sensory neurons innervating the lungs. *J Allergy Clin Immunol* **116**, 325-331, doi:10.1016/j.jaci.2005.04.005 (2005).

304 Hunter, D. D., Myers, A. C. & Undem, B. J. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. *Am J Respir Crit Care Med* **161**, 1985-1990, doi:10.1164/ajrccm.161.6.9908051 (2000).

305 Dinh, Q. T. *et al.* Nerve growth factor-induced substance P in capsaicin-insensitive vagal neurons innervating the lower mouse airway. *Clin Exp Allergy* **34**, 1474-1479, doi:10.1111/j.1365-2222.2004.02066.x (2004).

306 Costello, R. W., Fryer, A. D., Belmonte, K. E. & Jacoby, D. B. Effects of tachykinin NK1 receptor antagonists on vagal hyperreactivity and neuronal M2 muscarinic receptor function in antigen challenged guinea-pigs. *Br J Pharmacol* **124**, 267-276, doi:10.1038/sj.bjp.0701822 (1998).

307 Boot, J. D. *et al.* Effect of an NK1/NK2 receptor antagonist on airway responses and inflammation to allergen in asthma. *Am J Respir Crit Care Med* **175**, 450-457, doi:10.1164/rccm.200608-1186OC (2007).

308 Morteau, O., Lu, B., Gerard, C. & Gerard, N. P. Hemokinin 1 is a full agonist at the substance P receptor. *Nat Immunol* **2**, 1088, doi:10.1038/ni1201-1088 (2001).

309 Han, L. *et al.* Mrgprs on vagal sensory neurons contribute to bronchoconstriction and airway hyper-responsiveness. *Nat Neurosci* **21**, 324-328, doi:10.1038/s41593-018-0074-8 (2018).

310 Lundberg, J. M., Franco-Cereceda, A., Hua, X., Hokfelt, T. & Fischer, J. A. Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur J Pharmacol* **108**, 315-319 (1985).

311 Martling, C. R., Saria, A., Fischer, J. A., Hokfelt, T. & Lundberg, J. M. Calcitonin gene-related peptide and the lung: neuronal coexistence with substance P, release by capsaicin and vasodilatory effect. *Regul Pept* **20**, 125-139 (1988).

312 Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. & Evans, R. M. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* **298**, 240-244 (1982).

313 Rosenfeld, M. G. *et al.* Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* **304**, 129-135 (1983).

314 Russo, A. F., Nelson, C., Roos, B. A. & Rosenfeld, M. G. Differential regulation of the coexpressed calcitonin/alpha-CGRP and beta-CGRP neuroendocrine genes. *J Biol Chem* **263**, 5-8 (1988).

315 Uddman, R., Luts, A. & Sundler, F. Occurrence and distribution of calcitonin gene-related peptide in the mammalian respiratory tract and middle ear. *Cell Tissue Res* **241**, 551-555 (1985).

316 Springall, D. R. *et al.* Retrograde tracing shows that CGRP-immunoreactive nerves of rat trachea and lung originate from vagal and dorsal root ganglia. *J Auton Nerv Syst* **20**, 155-166 (1987).

317 Verastegui, C., Prada Oliveira, A., Fernandez-Vivero, J., Romero, A. & de Castro, J. M. Calcitonin gene-related peptide immunoreactivity in adult mouse lung. *Eur J Histochem* **41**, 119-126 (1997).

318 Kajekar, R. & Myers, A. C. Calcitonin gene-related peptide affects synaptic and membrane properties of bronchial parasympathetic neurons. *Respir Physiol Neurobiol* **160**, 28-36, doi:10.1016/j.resp.2007.07.010 (2008).

319 Keith, I. M., Pelto-Huikko, M., Schalling, M. & Hokfelt, T. Calcitonin gene-related peptide and its mRNA in pulmonary neuroendocrine cells and ganglia. *Histochemistry* **96**, 311-315 (1991).

320 Mason, R. T. *et al.* Release of the predicted calcitonin gene-related peptide from cultured rat trigeminal ganglion cells. *Nature* **308**, 653-655 (1984).

321 Kroll, F., Karlsson, J. A., Lundberg, J. M. & Persson, C. G. Capsaicin-induced bronchoconstriction and neuropeptide release in guinea pig perfused lungs. *J Appl Physiol (1985)* **68**, 1679-1687, doi:10.1152/jappl.1990.68.4.1679 (1990).

322 Hua, X. Y. & Yaksh, T. L. Pharmacology of the effects of bradykinin, serotonin, and histamine on the release of calcitonin gene-related peptide from C-fiber terminals in the rat trachea. *J Neurosci* **13**, 1947-1953 (1993).

323 Hua, X. Y., Jinno, S., Back, S. M., Tam, E. K. & Yaksh, T. L. Multiple mechanisms for the effects of capsaicin, bradykinin and nicotine on CGRP release from tracheal afferent nerves: role of prostaglandins, sympathetic nerves and mast cells. *Neuropharmacology* **33**, 1147-1154 (1994).

324 Eberhardt, M. *et al.* H2S and NO cooperatively regulate vascular tone by activating a neuroendocrine HNO-TRPA1-CGRP signalling pathway. *Nat Commun* **5**, 4381, doi:10.1038/ncomms5381 (2014).

325 Ushio, N., Dai, Y., Wang, S., Fukuoka, T. & Noguchi, K. Transient receptor potential channel A1 involved in calcitonin gene-related peptide release in neurons. *Neural Regen Res* **8**, 3013-3019, doi:10.3969/j.issn.1673-5374.2013.32.004 (2013).

326 Xiao, Y., Richter, J. A. & Hurley, J. H. Release of glutamate and CGRP from trigeminal ganglion neurons: Role of calcium channels and 5-HT1 receptor signaling. *Mol Pain* **4**, 12, doi:10.1186/1744-8069-4-12 (2008).

327 Durham, P. L., Sharma, R. V. & Russo, A. F. Repression of the calcitonin gene-related peptide promoter by 5-HT1 receptor activation. *J Neurosci* **17**, 9545-9553 (1997).

328 Booe, J. M. *et al.* Structural Basis for Receptor Activity-Modifying Protein-Dependent Selective Peptide Recognition by a G Protein-Coupled Receptor. *Mol Cell* **58**, 1040-1052, doi:10.1016/j.molcel.2015.04.018 (2015).

329 Boudard, F. & Bastide, M. Inhibition of mouse T-cell proliferation by CGRP and VIP: effects of these neuropeptides on IL-2 production and cAMP synthesis. *J Neurosci Res* **29**, 29-41, doi:10.1002/jnr.490290104 (1991).

330 Wang, F., Millet, I., Bottomly, K. & Vignery, A. Calcitonin gene-related peptide inhibits interleukin 2 production by murine T lymphocytes. *J Biol Chem* **267**, 21052-21057 (1992).
331 Kubota, M. *et al.* Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cells. *Biochem Biophys Res Commun* **132**, 88-94 (1985).
332 Anderson, L. E. & Seybold, V. S. Calcitonin gene-related peptide regulates gene transcription in primary afferent neurons. *J Neurochem* **91**, 1417-1429, doi:10.1111/j.1471-4159.2004.02833.x (2004).
333 Katayama, M. *et al.* Catabolism of calcitonin gene-related peptide and substance P by neutral endopeptidase. *Peptides* **12**, 563-567 (1991).
334 Walls, A. F. *et al.* Human mast cell tryptase attenuates the vasodilator activity of calcitonin gene-related peptide. *Biochem Pharmacol* **43**, 1243-1248 (1992).
335 Tam, E. K. & Caughey, G. H. Degradation of airway neuropeptides by human lung tryptase. *Am J Respir Cell Mol Biol* **3**, 27-32, doi:10.1165/ajrcmb/3.1.27 (1990).
336 Balkowiec-Iskra, E., Vermehren-Schmaedick, A. & Balkowiec, A. Tumor necrosis factor-alpha increases brain-derived neurotrophic factor expression in trigeminal ganglion neurons in an activity-dependent manner. *Neuroscience* **180**, 322-333, doi:10.1016/j.neuroscience.2011.02.028 (2011).
337 Li, W., Hou, L., Hua, Z. & Wang, X. Interleukin-1beta induces beta-calcitonin gene-related peptide secretion in human type II alveolar epithelial cells. *FASEB J* **18**, 1603-1605, doi:10.1096/fj.04-1737fje (2004).
338 Hou, L., Li, W. & Wang, X. Mechanism of interleukin-1 beta-induced calcitonin gene-related peptide production from dorsal root ganglion neurons of neonatal rats. *J Neurosci Res* **73**, 188-197, doi:10.1002/jnr.10651 (2003).
339 Park, K. A. *et al.* Signaling pathways that mediate nerve growth factor-induced increase in expression and release of calcitonin gene-related peptide from sensory neurons. *Neuroscience* **171**, 910-923, doi:10.1016/j.neuroscience.2010.09.027 (2010).
340 Ramer, M. S., Bradbury, E. J., Michael, G. J., Lever, I. J. & McMahon, S. B. Glial cell line-derived neurotrophic factor increases calcitonin gene-related peptide immunoreactivity in sensory and motoneurons in vivo. *Eur J Neurosci* **18**, 2713-2721 (2003).
341 Kay, A. B. *et al.* Airway expression of calcitonin gene-related peptide in T-cell peptide-induced late asthmatic reactions in atopics. *Allergy* **62**, 495-503, doi:10.1111/j.1398-9995.2007.01342.x (2007).
342 Dakhama, A. *et al.* Regulation of airway hyperresponsiveness by calcitonin gene-related peptide in allergen sensitized and challenged mice. *Am J Respir Crit Care Med* **165**, 1137-1144, doi:10.1164/ajrccm.165.8.2109058 (2002).
343 Tsukiji, J. *et al.* Long-term induction of beta-CGRP mRNA in rat lungs by allergic inflammation. *Life Sci* **76**, 163-177, doi:10.1016/j.lfs.2004.05.038 (2004).
344 Le, D. D. *et al.* Steroid Treatment Reduces Allergic Airway Inflammation and Does Not Alter the Increased Numbers of Dendritic Cells and Calcitonin Gene-Related Peptide-Expressing Neurons in Airway Sensory Ganglia. *Neuroimmunomodulation* **23**, 18-26, doi:10.1159/000440622 (2016).
345 Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R. & MacIntyre, I. Calcitonin gene-related peptide is a potent vasodilator. *Nature* **313**, 54-56 (1985).
346 McCormack, D. G., Mak, J. C., Coupe, M. O. & Barnes, P. J. Calcitonin gene-related peptide vasodilation of human pulmonary vessels. *J Appl Physiol (1985)* **67**, 1265-1270, doi:10.1152/jappl.1989.67.3.1265 (1989).

347 Brain, S. D. & Williams, T. J. Inflammatory oedema induced by synergism between calcitonin gene-related peptide (CGRP) and mediators of increased vascular permeability. *Br J Pharmacol* **86**, 855-860 (1985).
348 Gamse, R. & Saria, A. Potentiation of tachykinin-induced plasma protein extravasation by calcitonin gene-related peptide. *Eur J Pharmacol* **114**, 61-66 (1985).
349 Brokaw, J. J. & White, G. W. Calcitonin gene-related peptide potentiates substance P-induced plasma extravasation in the rat trachea. *Lung* **170**, 85-93 (1992).
350 Brain, S. D. & Williams, T. J. Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature* **335**, 73-75, doi:10.1038/335073a0 (1988).
351 Nong, Y. H., Titus, R. G., Ribeiro, J. M. & Remold, H. G. Peptides encoded by the calcitonin gene inhibit macrophage function. *J Immunol* **143**, 45-49 (1989).
352 Rochlitzer, S. *et al.* The neuropeptide calcitonin gene-related peptide affects allergic airway inflammation by modulating dendritic cell function. *Clin Exp Allergy* **41**, 1609-1621, doi:10.1111/j.1365-2222.2011.03822.x (2011).
353 Carucci, J. A. *et al.* Calcitonin gene-related peptide decreases expression of HLA-DR and CD86 by human dendritic cells and dampens dendritic cell-driven T cell-proliferative responses via the type I calcitonin gene-related peptide receptor. *J Immunol* **164**, 3494-3499 (2000).
354 Fox, F. E. *et al.* Calcitonin gene-related peptide inhibits proliferation and antigen presentation by human peripheral blood mononuclear cells: effects on B7, interleukin 10, and interleukin 12. *J Invest Dermatol* **108**, 43-48 (1997).
355 Hastings, R. H. & Hua, X. Y. Expression of calcitonin gene-related peptide by cultured rat alveolar type II cells. *Am J Respir Cell Mol Biol* **13**, 563-569, doi:10.1165/ajrcmb.13.5.7576692 (1995).
356 Wang, W. *et al.* Endogenous calcitonin gene-related peptide protects human alveolar epithelial cells through protein kinase Cepsilon and heat shock protein. *J Biol Chem* **280**, 20325-20330, doi:10.1074/jbc.M413864200 (2005).
357 Kawanami, Y. *et al.* Calcitonin gene-related peptide stimulates proliferation of alveolar epithelial cells. *Respir Res* **10**, 8, doi:10.1186/1465-9921-10-8 (2009).
358 Sanghavi, J. N. *et al.* Migration of human and guinea pig airway epithelial cells in response to calcitonin gene-related peptide. *Am J Respir Cell Mol Biol* **11**, 181-187, doi:10.1165/ajrcmb.11.2.8049078 (1994).
359 Zhou, Y. *et al.* Calcitonin gene-related peptide promotes the wound healing of human bronchial epithelial cells via PKC and MAPK pathways. *Regul Pept* **184**, 22-29, doi:10.1016/j.regpep.2013.03.020 (2013).
360 Bhogal, R., Sheldrick, R. L., Coleman, R. A., Smith, D. M. & Bloom, S. R. The effects of IAPP and CGRP on guinea pig tracheal smooth muscle in vitro. *Peptides* **15**, 1243-1247 (1994).
361 Cadieux, A., Lanoue, C., Sirois, P. & Barabe, J. Carbamylcholine- and 5-hydroxytryptamine-induced contraction in rat isolated airways: inhibition by calcitonin gene-related peptide. *Br J Pharmacol* **101**, 193-199 (1990).
362 Gatto, C. *et al.* Calcitonin and CGRP block bombesin- and substance P-induced increases in airway tone. *J Appl Physiol (1985)* **66**, 573-577, doi:10.1152/jappl.1989.66.2.573 (1989).
363 Schworer, H., Schmidt, W. E., Katsoulis, S. & Creutzfeldt, W. Calcitonin gene-related peptide (CGRP) modulates cholinergic neurotransmission in the small intestine of man, pig and guinea-pig via presynaptic CGRP receptors. *Regul Pept* **36**, 345-358 (1991).

364 Kimura, I., Okazaki, M. & Nojima, H. Mutual dependence of calcitonin-gene related peptide and acetylcholine release in neuromuscular preparations. *Eur J Pharmacol* **330**, 123-128 (1997).
365 Aoki-Nagase, T. *et al.* Attenuation of antigen-induced airway hyperresponsiveness in CGRP-deficient mice. *Am J Physiol Lung Cell Mol Physiol* **283**, L963-970, doi:10.1152/ajplung.00130.2002 (2002).
366 Oh-hashi, Y. *et al.* Elevated sympathetic nervous activity in mice deficient in alphaCGRP. *Circ Res* **89**, 983-990 (2001).
367 Ayala, L. E. & Ahmed, T. Is there loss of protective muscarinic receptor mechanism in asthma? *Chest* **96**, 1285-1291 (1989).
368 Minette, P. A., Lammers, J. W., Dixon, C. M., McCusker, M. T. & Barnes, P. J. A muscarinic agonist inhibits reflex bronchoconstriction in normal but not in asthmatic subjects. *J Appl Physiol* **67**, 2461-2465 (1989).
369 Fryer, A. D. & Wills-Karp, M. Dysfunction of M2-muscarinic receptors in pulmonary parasympathetic nerves after antigen challenge. *J Appl Physiol* **71**, 2255-2261 (1991).
370 Elbon, C. L., Jacoby, D. B. & Fryer, A. D. Pretreatment with an antibody to interleukin-5 prevents loss of pulmonary M2 muscarinic receptor function in antigen-challenged guinea pigs. *Am J Respir Cell Mol Biol* **12**, 320-328, doi:10.1165/ajrcmb.12.3.7873198 (1995).
371 Fryer, A. D. *et al.* Antibody to VLA-4, but not to L-selectin, protects neuronal M2 muscarinic receptors in antigen-challenged guinea pig airways. *J Clin Invest* **99**, 2036-2044, doi:10.1172/JCI119372 (1997).
372 Fryer, A. D. *et al.* Neuronal eotaxin and the effects of CCR3 antagonist on airway hyperreactivity and M2 receptor dysfunction. *J Clin Invest* **116**, 228-236, doi:10.1172/JCI25423 (2006).
373 Costello, R. W. *et al.* Localization of eosinophils to airway nerves and effect on neuronal M2 muscarinic receptor function. *Am J Physiol* **273**, L93-103, doi:10.1152/ajplung.1997.273.1.L93 (1997).
374 Sawatzky, D. A. *et al.* Eosinophil adhesion to cholinergic nerves via ICAM-1 and VCAM-1 and associated eosinophil degranulation. *Am J Physiol Lung Cell Mol Physiol* **282**, L1279-1288, doi:10.1152/ajplung.00279.2001 (2002).
375 Jacoby, D. B., Gleich, G. J. & Fryer, A. D. Human eosinophil major basic protein is an endogenous allosteric antagonist at the inhibitory muscarinic M2 receptor. *J Clin Invest* **91**, 1314-1318 (1993).
376 Levi-Montalcini, R. & Angeletti, P. U. Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. *Dev Biol* **6**, 653-659 (1963).
377 Zaimis, E., Berk, L. & Callingham, B. A. Morphological, biochemical and functional changes in the sympathetic nervous system of rats treated with nerve growth factor-antiserum. *Nature* **206**, 1220-1222 (1965).
378 Frazier, W. A. *et al.* Mechanism of action of nerve growth factor and cyclic AMP on neurite outgrowth in embryonic chick sensory ganglia: demonstration of independent pathways of stimulation. *Proc Natl Acad Sci U S A* **70**, 2448-2452 (1973).
379 Campenot, R. B. Local control of neurite development by nerve growth factor. *Proc Natl Acad Sci U S A* **74**, 4516-4519 (1977).
380 Gundersen, R. W. & Barrett, J. N. Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. *Science* **206**, 1079-1080 (1979).

381 Shelton, D. L. & Reichardt, L. F. Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc Natl Acad Sci U S A* **81**, 7951-7955 (1984).

382 Korsching, S. & Thoenen, H. Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: correlation with density of sympathetic innervation. *Proc Natl Acad Sci U S A* **80**, 3513-3516 (1983).

383 Thoenen, H., Angeletti, P. U., Levi-Montalcini, R. & Kettler, R. Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine- -hydroxylase in the rat superior cervical ganglia. *Proc Natl Acad Sci U S A* **68**, 1598-1602 (1971).

384 Berger, E. A. & Shooter, E. M. Evidence for pro-beta-nerve growth factor, a biosynthetic precursor to beta-nerve growth factor. *Proc Natl Acad Sci U S A* **74**, 3647-3651 (1977).

385 Lim, K. C., Tyler, C. M., Lim, S. T., Giuliano, R. & Federoff, H. J. Proteolytic processing of proNGF is necessary for mature NGF regulated secretion from neurons. *Biochem Biophys Res Commun* **361**, 599-604, doi:10.1016/j.bbrc.2007.07.039 (2007).

386 Lee, R., Kermani, P., Teng, K. K. & Hempstead, B. L. Regulation of cell survival by secreted proneurotrophins. *Science* **294**, 1945-1948, doi:10.1126/science.1065057 (2001).

387 Sutter, A., Riopelle, R. J., Harris-Warrick, R. M. & Shooter, E. M. Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. *J Biol Chem* **254**, 5972-5982 (1979).

388 Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V. & Parada, L. F. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**, 554-558 (1991).

389 Klein, R., Jing, S. Q., Nanduri, V., O'Rourke, E. & Barbacid, M. The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65**, 189-197 (1991).

390 Kaplan, D. R., Martin-Zanca, D. & Parada, L. F. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350**, 158-160, doi:10.1038/350158a0 (1991).

391 Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. & Brugge, J. S. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**, 1031-1040 (1992).

392 Wood, K. W., Sarnecki, C., Roberts, T. M. & Blenis, J. ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* **68**, 1041-1050 (1992).

393 Gomez, N. & Cohen, P. Dissection of the protein kinase cascade by which nerve growth factor activates MAP kinases. *Nature* **353**, 170-173, doi:10.1038/353170a0 (1991).

394 Segal, R. A. Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci* **26**, 299-330, doi:10.1146/annurev.neuro.26.041002.131421 (2003).

395 Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. & Shooter, E. M. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature* **325**, 593-597, doi:10.1038/325593a0 (1987).

396 Johnson, D. *et al.* Expression and structure of the human NGF receptor. *Cell* **47**, 545-554 (1986).

397 Carter, B. D. *et al.* Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. *Science* **272**, 542-545 (1996).

398 Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. & He, Z. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* **420**, 74-78, doi:10.1038/nature01176 (2002).
399 Yoon, S. O., Casaccia-Bonnefil, P., Carter, B. & Chao, M. V. Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival. *J Neurosci* **18**, 3273-3281 (1998).
400 Lad, S. P., Peterson, D. A., Bradshaw, R. A. & Neet, K. E. Individual and combined effects of TrkA and p75NTR nerve growth factor receptors. A role for the high affinity receptor site. *J Biol Chem* **278**, 24808-24817, doi:10.1074/jbc.M212270200 (2003).
401 Wehrman, T. *et al.* Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors. *Neuron* **53**, 25-38, doi:10.1016/j.neuron.2006.09.034 (2007).
402 Barrett, G. L. & Bartlett, P. F. The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc Natl Acad Sci U S A* **91**, 6501-6505 (1994).
403 Frade, J. M., Rodriguez-Tebar, A. & Barde, Y. A. Induction of cell death by endogenous nerve growth factor through its p75 receptor. *Nature* **383**, 166-168, doi:10.1038/383166a0 (1996).
404 Kassel, O. *et al.* Local increase in the number of mast cells and expression of nerve growth factor in the bronchus of asthmatic patients after repeated inhalation of allergen at low-dose. *Clin Exp Allergy* **31**, 1432-1440 (2001).
405 Olgart Hoglund, C. *et al.* Nerve growth factor levels and localisation in human asthmatic bronchi. *Eur Respir J* **20**, 1110-1116 (2002).
406 Hu, C., Wedde-Beer, K., Auais, A., Rodriguez, M. M. & Piedimonte, G. Nerve growth factor and nerve growth factor receptors in respiratory syncytial virus-infected lungs. *Am J Physiol Lung Cell Mol Physiol* **283**, L494-502, doi:10.1152/ajplung.00414.2001 (2002).
407 Fox, A. J., Patel, H. J., Barnes, P. J. & Belvisi, M. G. Release of nerve growth factor by human pulmonary epithelial cells: role in airway inflammatory diseases. *Eur J Pharmacol* **424**, 159-162 (2001).
408 Pons, F. *et al.* Nerve growth factor secretion by human lung epithelial A549 cells in pro- and anti-inflammatory conditions. *Eur J Pharmacol* **428**, 365-369 (2001).
409 Lindholm, D., Heumann, R., Meyer, M. & Thoenen, H. Interleukin-1 regulates synthesis of nerve growth factor in non-neuronal cells of rat sciatic nerve. *Nature* **330**, 658-659, doi:10.1038/330658a0 (1987).
410 Hattori, A. *et al.* Tumor necrosis factor stimulates the synthesis and secretion of biologically active nerve growth factor in non-neuronal cells. *J Biol Chem* **268**, 2577-2582 (1993).
411 Olgart, C. & Frossard, N. Human lung fibroblasts secrete nerve growth factor: effect of inflammatory cytokines and glucocorticoids. *Eur Respir J* **18**, 115-121 (2001).
412 Freund, V. *et al.* Upregulation of nerve growth factor expression by human airway smooth muscle cells in inflammatory conditions. *Eur Respir J* **20**, 458-463 (2002).
413 Bonini, S. *et al.* Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci U S A* **93**, 10955-10960 (1996).
414 Virchow, J. C. *et al.* Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. *Am J Respir Crit Care Med* **158**, 2002-2005, doi:10.1164/ajrccm.158.6.9803023 (1998).

415 Noga, O., Hanf, G., Schaper, C., O'Connor, A. & Kunkel, G. The influence of inhalative corticosteroids on circulating Nerve Growth Factor, Brain-Derived Neurotrophic Factor and Neurotrophin-3 in allergic asthmatics. *Clin Exp Allergy* **31**, 1906-1912 (2001).

416 Winter, J., Forbes, C. A., Sternberg, J. & Lindsay, R. M. Nerve growth factor (NGF) regulates adult rat cultured dorsal root ganglion neuron responses to the excitotoxin capsaicin. *Neuron* **1**, 973-981 (1988).

417 Frossard, N., Naline, E., Olgart Hoglund, C., Georges, O. & Advenier, C. Nerve growth factor is released by IL-1beta and induces hyperresponsiveness of the human isolated bronchus. *Eur Respir J* **26**, 15-20, doi:10.1183/09031936.05.00047804 (2005).

418 Wu, Z. X. & Dey, R. D. Nerve growth factor-enhanced airway responsiveness involves substance P in ferret intrinsic airway neurons. *Am J Physiol Lung Cell Mol Physiol* **291**, L111-118, doi:10.1152/ajplung.00377.2005 (2006).

419 de Vries, A., Dessing, M. C., Engels, F., Henricks, P. A. & Nijkamp, F. P. Nerve growth factor induces a neurokinin-1 receptor- mediated airway hyperresponsiveness in guinea pigs. *Am J Respir Crit Care Med* **159**, 1541-1544, doi:10.1164/ajrccm.159.5.9808058 (1999).

420 Winston, J., Toma, H., Shenoy, M. & Pasricha, P. J. Nerve growth factor regulates VR-1 mRNA levels in cultures of adult dorsal root ganglion neurons. *Pain* **89**, 181-186 (2001).

421 Mamet, J., Lazdunski, M. & Voilley, N. How nerve growth factor drives physiological and inflammatory expressions of acid-sensing ion channel 3 in sensory neurons. *J Biol Chem* **278**, 48907-48913, doi:10.1074/jbc.M309468200 (2003).

422 Ramer, M. S., Bradbury, E. J. & McMahon, S. B. Nerve growth factor induces P2X(3) expression in sensory neurons. *J Neurochem* **77**, 864-875 (2001).

423 Weigand, L. A., Kwong, K. & Myers, A. C. The Effects of Nerve Growth Factor on Nicotinic Synaptic Transmission in Mouse Airway Parasympathetic Neurons. *Am J Respir Cell Mol Biol* **53**, 443-449, doi:10.1165/rcmb.2014-0280OC (2015).

424 De Vries, A. *et al.* Antibodies directed against nerve growth factor inhibit the acute bronchoconstriction due to allergen challenge in guinea-pigs. *Clin Exp Allergy* **32**, 325-328 (2002).

425 Kerzel, S. *et al.* Pan-neurotrophin receptor p75 contributes to neuronal hyperreactivity and airway inflammation in a murine model of experimental asthma. *Am J Respir Cell Mol Biol* **28**, 170-178, doi:10.1165/rcmb.4811 (2003).

426 Liu, Y. *et al.* Nerve growth factor mediated SH2-Bbeta/Akt signal pathway activated in allergic airway challenge in mice. *Respirology* **15**, 80-87, doi:10.1111/j.1440-1843.2009.01648.x (2010).

427 Chen, Y. L., Huang, H. Y., Lee, C. C. & Chiang, B. L. Small interfering RNA targeting nerve growth factor alleviates allergic airway hyperresponsiveness. *Mol Ther Nucleic Acids* **3**, e158, doi:10.1038/mtna.2014.11 (2014).

428 Nassenstein, C. *et al.* Neuroimmune crosstalk in asthma: dual role of the neurotrophin receptor p75NTR. *J Allergy Clin Immunol* **120**, 1089-1096, doi:10.1016/j.jaci.2007.07.007 (2007).

429 Yang, Y. G., Tian, W. M., Zhang, H., Li, M. & Shang, Y. X. Nerve growth factor exacerbates allergic lung inflammation and airway remodeling in a rat model of chronic asthma. *Exp Ther Med* **6**, 1251-1258, doi:10.3892/etm.2013.1284 (2013).

430 Leon, A. *et al.* Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci U S A* **91**, 3739-3743 (1994).

431 Lambiase, A. *et al.* Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *J Allergy Clin Immunol* **100**, 408-414 (1997).

432 Ehrhard, P. B., Erb, P., Graumann, U. & Otten, U. Expression of nerve growth factor and nerve growth factor receptor tyrosine kinase Trk in activated CD4-positive T-cell clones. *Proc Natl Acad Sci U S A* **90**, 10984-10988 (1993).

433 Xiang, Z. & Nilsson, G. IgE receptor-mediated release of nerve growth factor by mast cells. *Clin Exp Allergy* **30**, 1379-1386 (2000).

434 Mazurek, N., Weskamp, G., Erne, P. & Otten, U. Nerve growth factor induces mast cell degranulation without changing intracellular calcium levels. *FEBS Lett* **198**, 315-320 (1986).

435 Horigome, K., Pryor, J. C., Bullock, E. D. & Johnson, E. M., Jr. Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. *J Biol Chem* **268**, 14881-14887 (1993).

436 Pearce, F. L. & Thompson, H. L. Some characteristics of histamine secretion from rat peritoneal mast cells stimulated with nerve growth factor. *J Physiol* **372**, 379-393 (1986).

437 Noga, O. *et al.* The production, storage and release of the neurotrophins nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 by human peripheral eosinophils in allergics and non-allergics. *Clin Exp Allergy* **33**, 649-654 (2003).

438 Hahn, C., Islamian, A. P., Renz, H. & Nockher, W. A. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. *J Allergy Clin Immunol* **117**, 787-794, doi:10.1016/j.jaci.2005.12.1339 (2006).

439 Matsuda, H., Coughlin, M. D., Bienenstock, J. & Denburg, J. A. Nerve growth factor promotes human hemopoietic colony growth and differentiation. *Proc Natl Acad Sci U S A* **85**, 6508-6512 (1988).

440 Hamada, A., Watanabe, N., Ohtomo, H. & Matsuda, H. Nerve growth factor enhances survival and cytotoxic activity of human eosinophils. *Br J Haematol* **93**, 299-302 (1996).

441 Solomon, A. *et al.* Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *J Allergy Clin Immunol* **102**, 454-460 (1998).

442 Kilic, A. *et al.* Nerve growth factor induces type III collagen production in chronic allergic airway inflammation. *J Allergy Clin Immunol* **128**, 1058-1066 e1051-1054, doi:10.1016/j.jaci.2011.06.017 (2011).

443 Freund-Michel, V., Bertrand, C. & Frossard, N. TrkA signalling pathways in human airway smooth muscle cell proliferation. *Cell Signal* **18**, 621-627, doi:10.1016/j.cellsig.2005.06.007 (2006).

444 Leibrock, J. *et al.* Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341**, 149-152, doi:10.1038/341149a0 (1989).

445 Hofer, M. M. & Barde, Y. A. Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* **331**, 261-262, doi:10.1038/331261a0 (1988).

446 Conover, J. C. *et al.* Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* **375**, 235-238, doi:10.1038/375235a0 (1995).

447 Barde, Y. A., Lindsay, R. M., Monard, D. & Thoenen, H. New factor released by cultured glioma cells supporting survival and growth of sensory neurones. *Nature* **274**, 818 (1978).

448 Jones, K. R., Farinas, I., Backus, C. & Reichardt, L. F. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76**, 989-999 (1994).

449 Ernfors, P., Lee, K. F. & Jaenisch, R. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. *Nature* **368**, 147-150, doi:10.1038/368147a0 (1994).
450 Carroll, P., Lewin, G. R., Koltzenburg, M., Toyka, K. V. & Thoenen, H. A role for BDNF in mechanosensation. *Nat Neurosci* **1**, 42-46, doi:10.1038/242 (1998).
451 Perez-Pinera, P. *et al.* Characterization of sensory deficits in TrkB knockout mice. *Neurosci Lett* **433**, 43-47, doi:10.1016/j.neulet.2007.12.035 (2008).
452 Lindsay, R. M., Thoenen, H. & Barde, Y. A. Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. *Dev Biol* **112**, 319-328 (1985).
453 Koliatsos, V. E., Clatterbuck, R. E., Winslow, J. W., Cayouette, M. H. & Price, D. L. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo. *Neuron* **10**, 359-367 (1993).
454 Sendtner, M., Holtmann, B., Kolbeck, R., Thoenen, H. & Barde, Y. A. Brain-derived neurotrophic factor prevents the death of motoneurons in newborn rats after nerve section. *Nature* **360**, 757-759, doi:10.1038/360757a0 (1992).
455 Oppenheim, R. W., Yin, Q. W., Prevette, D. & Yan, Q. Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. *Nature* **360**, 755-757, doi:10.1038/360755a0 (1992).
456 Yan, Q., Elliott, J. & Snider, W. D. Brain-derived neurotrophic factor rescues spinal motor neurons from axotomy-induced cell death. *Nature* **360**, 753-755, doi:10.1038/360753a0 (1992).
457 Nagappan, G. *et al.* Control of extracellular cleavage of ProBDNF by high frequency neuronal activity. *Proc Natl Acad Sci U S A* **106**, 1267-1272, doi:10.1073/pnas.0807322106 (2009).
458 Qiao, L. Y. *et al.* Inflammation and activity augment brain-derived neurotrophic factor peripheral release. *Neuroscience* **318**, 114-121, doi:10.1016/j.neuroscience.2016.01.018 (2016).
459 Squinto, S. P. *et al.* trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65**, 885-893 (1991).
460 Klein, R. *et al.* The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66**, 395-403 (1991).
461 Soppet, D. *et al.* The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65**, 895-903 (1991).
462 Rodriguez-Tebar, A., Dechant, G. & Barde, Y. A. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4**, 487-492 (1990).
463 Je, H. S. *et al.* Role of pro-brain-derived neurotrophic factor (proBDNF) to mature BDNF conversion in activity-dependent competition at developing neuromuscular synapses. *Proc Natl Acad Sci U S A* **109**, 15924-15929, doi:10.1073/pnas.1207767109 (2012).
464 Klein, R., Conway, D., Parada, L. F. & Barbacid, M. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* **61**, 647-656 (1990).
465 Rodriguez-Tebar, A., Jeffrey, P. L., Thoenen, H. & Barde, Y. A. The survival of chick retinal ganglion cells in response to brain-derived neurotrophic factor depends on their embryonic age. *Dev Biol* **136**, 296-303 (1989).

466 Braun, A. *et al.* Cellular sources of enhanced brain-derived neurotrophic factor production in a mouse model of allergic inflammation. *Am J Respir Cell Mol Biol* **21**, 537-546, doi:10.1165/ajrcmb.21.4.3670 (1999).
467 Aravamudan, B., Thompson, M. A., Pabelick, C. M. & Prakash, Y. S. Mechanisms of BDNF regulation in asthmatic airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **311**, L270-279, doi:10.1152/ajplung.00414.2015 (2016).
468 Kerschensteiner, M. *et al.* Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med* **189**, 865-870 (1999).
469 Lieu, T. M., Myers, A. C., Meeker, S. & Undem, B. J. TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors. *Am J Physiol Lung Cell Mol Physiol* **302**, L941-948, doi:10.1152/ajplung.00366.2011 (2012).
470 Vohra, P. K. *et al.* TRPC3 regulates release of brain-derived neurotrophic factor from human airway smooth muscle. *Biochim Biophys Acta* **1833**, 2953-2960, doi:10.1016/j.bbamcr.2013.07.019 (2013).
471 Watanabe, T. *et al.* Brain-Derived Neurotrophic Factor Expression in Asthma. Association with Severity and Type 2 Inflammatory Processes. *Am J Respir Cell Mol Biol* **53**, 844-852, doi:10.1165/rcmb.2015-0015OC (2015).
472 Muller, G. C. *et al.* Plasma brain-derived neurotrophic factor levels are associated with clinical severity in school age children with asthma. *Clin Exp Allergy* **40**, 1755-1759, doi:10.1111/j.1365-2222.2010.03618.x (2010).
473 Aravamudan, B., Thompson, M., Pabelick, C. & Prakash, Y. S. Brain-derived neurotrophic factor induces proliferation of human airway smooth muscle cells. *J Cell Mol Med* **16**, 812-823, doi:10.1111/j.1582-4934.2011.01356.x (2012).
474 Prakash, Y. S., Iyanoye, A., Ay, B., Mantilla, C. B. & Pabelick, C. M. Neurotrophin effects on intracellular Ca2+ and force in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* **291**, L447-456, doi:10.1152/ajplung.00501.2005 (2006).
475 Prakash, Y. S., Thompson, M. A. & Pabelick, C. M. Brain-derived neurotrophic factor in TNF-alpha modulation of Ca2+ in human airway smooth muscle. *Am J Respir Cell Mol Biol* **41**, 603-611, doi:10.1165/rcmb.2008-0151OC (2009).
476 Ghosh, A., Carnahan, J. & Greenberg, M. E. Requirement for BDNF in activity-dependent survival of cortical neurons. *Science* **263**, 1618-1623 (1994).
477 Shieh, P. B., Hu, S. C., Bobb, K., Timmusk, T. & Ghosh, A. Identification of a signaling pathway involved in calcium regulation of BDNF expression. *Neuron* **20**, 727-740 (1998).
478 Tao, X., Finkbeiner, S., Arnold, D. B., Shaywitz, A. J. & Greenberg, M. E. Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**, 709-726 (1998).
479 Frank, L., Ventimiglia, R., Anderson, K., Lindsay, R. M. & Rudge, J. S. BDNF down-regulates neurotrophin responsiveness, TrkB protein and TrkB mRNA levels in cultured rat hippocampal neurons. *Eur J Neurosci* **8**, 1220-1230 (1996).
480 Simonetti, M., Giniatullin, R. & Fabbretti, E. Mechanisms mediating the enhanced gene transcription of P2X3 receptor by calcitonin gene-related peptide in trigeminal sensory neurons. *J Biol Chem* **283**, 18743-18752, doi:10.1074/jbc.M800296200 (2008).
481 Winter, J. Brain derived neurotrophic factor, but not nerve growth factor, regulates capsaicin sensitivity of rat vagal ganglion neurones. *Neurosci Lett* **241**, 21-24 (1998).

482 Lieu, T. & Undem, B. J. Neuroplasticity in vagal afferent neurons involved in cough. *Pulm Pharmacol Ther* **24**, 276-279, doi:10.1016/j.pupt.2011.02.003 (2011).

483 Cohen-Cory, S. & Fraser, S. E. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature* **378**, 192-196, doi:10.1038/378192a0 (1995).

484 Alsina, B., Vu, T. & Cohen-Cory, S. Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. *Nat Neurosci* **4**, 1093-1101, doi:10.1038/nn735 (2001).

485 Zhou, X. *et al.* Brain-derived neurotrophic factor and trkB signaling in parasympathetic neurons: relevance to regulating alpha7-containing nicotinic receptors and synaptic function. *J Neurosci* **24**, 4340-4350, doi:10.1523/JNEUROSCI.0055-04.2004 (2004).

486 Zhang, X. & Poo, M. M. Localized synaptic potentiation by BDNF requires local protein synthesis in the developing axon. *Neuron* **36**, 675-688 (2002).

487 Noga, O. *et al.* Activation of the specific neurotrophin receptors TrkA, TrkB and TrkC influences the function of eosinophils. *Clin Exp Allergy* **32**, 1348-1354 (2002).

488 Pichel, J. G. *et al.* Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382**, 73-76, doi:10.1038/382073a0 (1996).

489 Moore, M. W. *et al.* Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382**, 76-79, doi:10.1038/382076a0 (1996).

490 Gianino, S., Grider, J. R., Cresswell, J., Enomoto, H. & Heuckeroth, R. O. GDNF availability determines enteric neuron number by controlling precursor proliferation. *Development* **130**, 2187-2198 (2003).

491 Mwizerwa, O. *et al.* Gdnf is mitogenic, neurotrophic, and chemoattractive to enteric neural crest cells in the embryonic colon. *Dev Dyn* **240**, 1402-1411, doi:10.1002/dvdy.22630 (2011).

492 Henderson, C. E. *et al.* GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062-1064 (1994).

493 Yan, Q., Matheson, C. & Lopez, O. T. In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* **373**, 341-344, doi:10.1038/373341a0 (1995).

494 Oppenheim, R. W. *et al.* Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* **373**, 344-346, doi:10.1038/373344a0 (1995).

495 Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A. & Davies, A. M. GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821-828 (1995).

496 Treanor, J. J. *et al.* Characterization of a multicomponent receptor for GDNF. *Nature* **382**, 80-83, doi:10.1038/382080a0 (1996).

497 Jing, S. *et al.* GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. *J Biol Chem* **272**, 33111-33117 (1997).

498 Cacalano, G. *et al.* GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. *Neuron* **21**, 53-62 (1998).

499 Trupp, M. *et al.* Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* **381**, 785-789, doi:10.1038/381785a0 (1996).

500 Durbec, P. *et al.* GDNF signalling through the Ret receptor tyrosine kinase. *Nature* **381**, 789-793, doi:10.1038/381789a0 (1996).

501 Jing, S. *et al.* GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. *Cell* **85**, 1113-1124 (1996).
502 Lieu, T., Kollarik, M., Myers, A. C. & Undem, B. J. Neurotrophin and GDNF family ligand receptor expression in vagal sensory nerve subtypes innervating the adult guinea pig respiratory tract. *Am J Physiol Lung Cell Mol Physiol* **300**, L790-798, doi:10.1152/ajplung.00449.2010 (2011).
503 Tollet, J., Everett, A. W. & Sparrow, M. P. Development of neural tissue and airway smooth muscle in fetal mouse lung explants: a role for glial-derived neurotrophic factor in lung innervation. *Am J Respir Cell Mol Biol* **26**, 420-429, doi:10.1165/ajrcmb.26.4.4713 (2002).
504 Ogun-Muyiwa, P., Helliwell, R., McIntyre, P. & Winter, J. Glial cell line derived neurotrophic factor (GDNF) regulates VR1 and substance P in cultured sensory neurons. *Neuroreport* **10**, 2107-2111 (1999).
505 Price, T. J. *et al.* Treatment of trigeminal ganglion neurons in vitro with NGF, GDNF or BDNF: effects on neuronal survival, neurochemical properties and TRPV1-mediated neuropeptide secretion. *BMC Neurosci* **6**, 4, doi:10.1186/1471-2202-6-4 (2005).
506 Amaya, F. *et al.* NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. *Eur J Neurosci* **20**, 2303-2310, doi:10.1111/j.1460-9568.2004.03701.x (2004).
507 Ribchester, R. R., Thomson, D., Haddow, L. J. & Ushkaryov, Y. A. Enhancement of spontaneous transmitter release at neonatal mouse neuromuscular junctions by the glial cell line-derived neurotrophic factor (GDNF). *J Physiol* **512 (Pt 3)**, 635-641 (1998).
508 Golden, J. P., DeMaro, J. A., Osborne, P. A., Milbrandt, J. & Johnson, E. M., Jr. Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. *Exp Neurol* **158**, 504-528, doi:10.1006/exnr.1999.7127 (1999).
509 Metzger, R. J., Klein, O. D., Martin, G. R. & Krasnow, M. A. The branching programme of mouse lung development. *Nature* **453**, 745-750, doi:10.1038/nature07005 (2008).
510 Burns, A. J. & Delalande, J. M. Neural crest cell origin for intrinsic ganglia of the developing chicken lung. *Dev Biol* **277**, 63-79, doi:10.1016/j.ydbio.2004.09.006 (2005).
511 Freem, L. J. *et al.* The intrinsic innervation of the lung is derived from neural crest cells as shown by optical projection tomography in Wnt1-Cre;YFP reporter mice. *J Anat* **217**, 651-664, doi:10.1111/j.1469-7580.2010.01295.x (2010).
512 Tollet, J., Everett, A. W. & Sparrow, M. P. Spatial and temporal distribution of nerves, ganglia, and smooth muscle during the early pseudoglandular stage of fetal mouse lung development. *Dev Dyn* **221**, 48-60, doi:10.1002/dvdy.1124 (2001).
513 Sparrow, M. P. & Lamb, J. P. Ontogeny of airway smooth muscle: structure, innervation, myogenesis and function in the fetal lung. *Respir Physiol Neurobiol* **137**, 361-372 (2003).
514 Burns, A. J., Thapar, N. & Barlow, A. J. Development of the neural crest-derived intrinsic innervation of the human lung. *Am J Respir Cell Mol Biol* **38**, 269-275, doi:10.1165/rcmb.2007-0246OC (2008).
515 Sparrow, M. P., Weichselbaum, M. & McCray, P. B. Development of the innervation and airway smooth muscle in human fetal lung. *Am J Respir Cell Mol Biol* **20**, 550-560, doi:10.1165/ajrcmb.20.4.3385 (1999).

516 Weichselbaum, M. & Sparrow, M. P. A confocal microscopic study of the formation of ganglia in the airways of fetal pig lung. *Am J Respir Cell Mol Biol* **21**, 607-620, doi:10.1165/ajrcmb.21.5.3721 (1999).
517 Sparrow, M. P., Warwick, S. P. & Everett, A. W. Innervation and function of the distal airways in the developing bronchial tree of fetal pig lung. *Am J Respir Cell Mol Biol* **13**, 518-525, doi:10.1165/ajrcmb.13.5.7576686 (1995).
518 Sparrow, M. P., Warwick, S. P. & Mitchell, H. W. Foetal airway motor tone in prenatal lung development of the pig. *Eur Respir J* **7**, 1416-1424 (1994).
519 Nassenstein, C. *et al.* Phenotypic distinctions between neural crest and placodal derived vagal C-fibres in mouse lungs. *J Physiol* **588**, 4769-4783, doi:10.1113/jphysiol.2010.195339 (2010).
520 Cadieux, A. *et al.* Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin treatment and surgical denervations. *Neuroscience* **19**, 605-627 (1986).
521 Salvi, E. & Renda, T. An immunohistochemical study on neurons and paraneurons of the pre- and post-natal chicken lung. *Arch Histol Cytol* **55**, 125-135 (1992).
522 Ratcliffe, E. M. *et al.* Netrin/DCC-mediated attraction of vagal sensory axons to the fetal mouse gut. *J Comp Neurol* **498**, 567-580, doi:10.1002/cne.21027 (2006).
523 Freem, L. J., Delalande, J. M., Campbell, A. M., Thapar, N. & Burns, A. J. Lack of organ specific commitment of vagal neural crest cell derivatives as shown by back-transplantation of GFP chicken tissues. *Int J Dev Biol* **56**, 245-254, doi:10.1387/ijdb.113438lf (2012).
524 Emanuilov, A. I., Shilkin, V. V., Nozdrachev, A. D. & Masliukov, P. M. Afferent innervation of the trachea during postnatal development. *Auton Neurosci* **120**, 68-72, doi:10.1016/j.autneu.2005.04.006 (2005).
525 Pan, J., Yeger, H. & Cutz, E. Innervation of pulmonary neuroendocrine cells and neuroepithelial bodies in developing rabbit lung. *J Histochem Cytochem* **52**, 379-389, doi:10.1177/002215540405200309 (2004).
526 Hunter, D. D., Wu, Z. & Dey, R. D. Sensory neural responses to ozone exposure during early postnatal development in rat airways. *Am J Respir Cell Mol Biol* **43**, 750-757, doi:10.1165/rcmb.2009-0191OC (2010).
527 Arakawa, H. *et al.* Effect of maturation on histamine-induced airflow obstruction and airway microvascular leakage in guinea pig airways. *Eur J Pharmacol* **215**, 51-56 (1992).
528 Zellner, L. C., Brundage, K. M., Hunter, D. D. & Dey, R. D. Early Postnatal Ozone Exposure Alters Rat Nodose and Jugular Sensory Neuron Development. *Toxicol Environ Chem* **93**, 2055-2071, doi:10.1080/02772248.2011.610882 (2011).
529 Patel, K. R., Bai, Y., Trieu, K. G., Barrios, J. & Ai, X. Targeting acetylcholine receptor M3 prevents the progression of airway hyperreactivity in a mouse model of childhood asthma. *FASEB J* **31**, 4335-4346, doi:10.1096/fj.201700186R (2017).
530 Hodgson, P. E., Rehder, K. & Hatch, D. J. Maturation of porcine tracheal cholinergic and inhibitory non-adrenergic, non-cholinergic innervation. *Pediatr Pulmonol* **23**, 354-361 (1997).
531 Tokuyama, K. *et al.* Attenuation of tachykinin-induced airflow obstruction and microvascular leakage in immature airways. *Br J Pharmacol* **108**, 23-29 (1993).
532 Buttery, L. D. *et al.* Early abundance of nerves containing NO synthase in the airways of newborn pigs and subsequent decrease with age. *Neurosci Lett* **201**, 219-222 (1995).

533 Kajekar, R. *et al.* Early postnatal exposure to allergen and ozone leads to hyperinnervation of the pulmonary epithelium. *Respir Physiol Neurobiol* **155**, 55-63, doi:10.1016/j.resp.2006.03.002 (2007).
534 Moore, B. D., Hyde, D. M., Miller, L. A., Wong, E. M. & Schelegle, E. S. Persistence of serotonergic enhancement of airway response in a model of childhood asthma. *Am J Respir Cell Mol Biol* **51**, 77-85, doi:10.1165/rcmb.2013-0387OC (2014).
535 Aven, L. *et al.* An NT4/TrkB-dependent increase in innervation links early-life allergen exposure to persistent airway hyperreactivity. *FASEB J* **28**, 897-907, doi:10.1096/fj.13-238212 (2014).
536 Wu, Z. X. *et al.* Prenatal and early, but not late, postnatal exposure of mice to sidestream tobacco smoke increases airway hyperresponsiveness later in life. *Environ Health Perspect* **117**, 1434-1440, doi:10.1289/ehp.0800511 (2009).
537 Wu, Z. X., Hunter, D. D., Batchelor, T. P. & Dey, R. D. Side-stream tobacco smoke-induced airway hyperresponsiveness in early postnatal period is involved nerve growth factor. *Respir Physiol Neurobiol* **223**, 1-8, doi:10.1016/j.resp.2015.11.009 (2016).
538 Saluzzo, S. *et al.* First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment. *Cell Rep* **18**, 1893-1905, doi:10.1016/j.celrep.2017.01.071 (2017).
539 Steer, C. A. *et al.* Group 2 innate lymphoid cell activation in the neonatal lung drives type 2 immunity and allergen sensitization. *J Allergy Clin Immunol* **140**, 593-595 e593, doi:10.1016/j.jaci.2016.12.984 (2017).
540 de Kleer, I. M. *et al.* Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung. *Immunity* **45**, 1285-1298, doi:10.1016/j.immuni.2016.10.031 (2016).
541 Yen, J. M. *et al.* Eosinophilia in very low birth weight infants. *Pediatr Neonatol* **51**, 116-123, doi:10.1016/S1875-9572(10)60021-6 (2010).
542 Yang, J. C., J.; Shim, SY.; Cho, S.; Park, E. The relationship between eosinophilia and bronchopulmonary dysplasia in premature infants at less than 34 weeks' gestation. *Korean J Pediatr* **57**, 171-177 (2014).
543 Sullivan, S. E. & Calhoun, D. A. Eosinophilia in the neonatal intensive care unit. *Clin Perinatol* **27**, 603-622, vi (2000).
544 Juul, S. E., Haynes, J. W. & McPherson, R. J. Evaluation of eosinophilia in hospitalized preterm infants. *J Perinatol* **25**, 182-188, doi:10.1038/sj.jp.7211226 (2005).
545 Ardini-Poleske, M. E. *et al.* LungMAP: The Molecular Atlas of Lung Development Program. *Am J Physiol Lung Cell Mol Physiol* **313**, L733-L740, doi:10.1152/ajplung.00139.2017 (2017).
546 Voorhorst, R. S. F. V., H.; Leupen, M; Lyklema, A. The house-dust mite (Dermatophagoides pteronyssinus) and the allergens it produces. Identity with the house-dust allergen. . *The Journal of Allergy* **39** (1967).
547 Woodcock, A. *et al.* Control of exposure to mite allergen and allergen-impermeable bed covers for adults with asthma. *N Engl J Med* **349**, 225-236, doi:10.1056/NEJMoa023175 (2003).
548 Gregory, L. G. *et al.* Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clin Exp Allergy* **39**, 1597-1610, doi:10.1111/j.1365-2222.2009.03302.x (2009).
549 Martin, T. R., Gerard, N. P., Galli, S. J. & Drazen, J. M. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl Physiol (1985)* **64**, 2318-2323, doi:10.1152/jappl.1988.64.6.2318 (1988).

550 Zhu, W. & Gilmour, M. I. Comparison of allergic lung disease in three mouse strains after systemic or mucosal sensitization with ovalbumin antigen. *Immunogenetics* **61**, 199-207, doi:10.1007/s00251-008-0353-8 (2009).
551 Takeda, K., Haczku, A., Lee, J. J., Irvin, C. G. & Gelfand, E. W. Strain dependence of airway hyperresponsiveness reflects differences in eosinophil localization in the lung. *Am J Physiol Lung Cell Mol Physiol* **281**, L394-402, doi:10.1152/ajplung.2001.281.2.L394 (2001).
552 Ochkur, S. I. *et al.* Coexpression of IL-5 and eotaxin-2 in mice creates an eosinophil-dependent model of respiratory inflammation with characteristics of severe asthma. *J Immunol* **178**, 7879-7889 (2007).
553 Lee, N. A. *et al.* Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J Immunol* **158**, 1332-1344 (1997).
554 Doyle, A. D. *et al.* Expression of the secondary granule proteins major basic protein 1 (MBP-1) and eosinophil peroxidase (EPX) is required for eosinophilopoiesis in mice. *Blood* **122**, 781-790, doi:10.1182/blood-2013-01-473405 (2013).
555 Kopf, M. *et al.* IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. *Immunity* **4**, 15-24 (1996).
556 Yu, C. *et al.* Targeted deletion of a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo. *J Exp Med* **195**, 1387-1395 (2002).
557 Nei, Y. *et al.* GATA-1 regulates the generation and function of basophils. *Proc Natl Acad Sci U S A* **110**, 18620-18625, doi:10.1073/pnas.1311668110 (2013).
558 Irvin, C. G. & Bates, J. H. Measuring the lung function in the mouse: the challenge of size. *Respir Res* **4**, 4 (2003).
559 Roberts, J. A., Rodger, I. W. & Thomson, N. C. Airway responsiveness to histamine in man: effect of atropine on in vivo and in vitro comparison. *Thorax* **40**, 261-267 (1985).
560 Bouhuys, A., Hunt, V. R., Kim, B. M. & Zapletal, A. Maximum expiratory flow rates in induced bronchoconstriction in man. *J Clin Invest* **48**, 1159-1168, doi:10.1172/JCI106073 (1969).
561 Polosa, R. *et al.* Effect of inhaled bradykinin on indices of airway responsiveness in asthmatic subjects. *Eur Respir J* **7**, 1490-1496 (1994).
562 Holroyde, M. C., Altounyan, R. E., Cole, M., Dixon, M. & Elliott, E. V. Bronchoconstriction produced in man by leukotrienes C and D. *Lancet* **2**, 17-18 (1981).
563 Cazzola, M. *et al.* Ketanserin, a new blocking agent of serotonin S2-receptors. Respiratory functional effects in chronic obstruction of the airways. *Chest* **92**, 863-866 (1987).
564 Beasley, R., Semprini, A. & Mitchell, E. A. Risk factors for asthma: is prevention possible? *Lancet* **386**, 1075-1085, doi:10.1016/S0140-6736(15)00156-7 (2015).
565 Lee, J. J. *et al.* Human versus mouse eosinophils: "that which we call an eosinophil, by any other name would stain as red". *J Allergy Clin Immunol* **130**, 572-584, doi:10.1016/j.jaci.2012.07.025 (2012).
566 Filley, W. V., Holley, K. E., Kephart, G. M. & Gleich, G. J. Identification by immunofluorescence of eosinophil granule major basic protein in lung tissues of patients with bronchial asthma. *Lancet* **2**, 11-16 (1982).
567 Broide, D. H. *et al.* Evidence of ongoing mast cell and eosinophil degranulation in symptomatic asthma airway. *J Allergy Clin Immunol* **88**, 637-648 (1991).

568 Stelts, D. *et al.* Eosinophils retain their granule major basic protein in a murine model of allergic pulmonary inflammation. *Am J Respir Cell Mol Biol* **18**, 463-470, doi:10.1165/ajrcmb.18.4.2957 (1998).
569 Satoh, J. & Yamakage, M. Desflurane induces airway contraction mainly by activating transient receptor potential A1 of sensory C-fibers. *J Anesth* **23**, 620-623, doi:10.1007/s00540-009-0786-8 (2009).
570 Kimball, C., Luo, J., Yin, S., Hu, H. & Dhaka, A. The Pore Loop Domain of TRPV1 Is Required for Its Activation by the Volatile Anesthetics Chloroform and Isoflurane. *Mol Pharmacol* **88**, 131-138, doi:10.1124/mol.115.098277 (2015).
571 Satoh, J. I. *et al.* Desflurane but not sevoflurane can increase lung resistance via tachykinin pathways. *Br J Anaesth* **102**, 704-713, doi:10.1093/bja/aep041 (2009).
572 Blaber, L. C., Fryer, A. D. & Maclagan, J. Neuronal muscarinic receptors attenuate vagally-induced contraction of feline bronchial smooth muscle. *Br J Pharmacol* **86**, 723-728 (1985).
573 Fryer, A. D. & Maclagan, J. Pancuronium and gallamine are antagonists for pre- and post-junctional muscarinic receptors in the guinea-pig lung. *Naunyn Schmiedebergs Arch Pharmacol* **335**, 367-371 (1987).
574 Quirion, R. *et al.* Autoradiographic distribution of substance P receptors in rat central nervous system. *Nature* **303**, 714-716 (1983).
575 Baluk, P., Thurston, G., Murphy, T. J., Bunnett, N. W. & McDonald, D. M. Neurogenic plasma leakage in mouse airways. *Br J Pharmacol* **126**, 522-528, doi:10.1038/sj.bjp.0702323 (1999).
576 Matsas, R., Kenny, A. J. & Turner, A. J. The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. *Biochem J* **223**, 433-440 (1984).
577 Wang, L., Zhou, R. & Xie, X. Tiotropium added to low- to medium-dose inhaled corticosteroids (ICS) versus low- to medium-dose ICS alone for adults with mild to moderate uncontrolled persistent asthma: A systematic review and meta-analysis. *J Asthma*, 1-10, doi:10.1080/02770903.2018.1424192 (2018).
578 Szefler, S. J. *et al.* A phase III randomized controlled trial of tiotropium add-on therapy in children with severe symptomatic asthma. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2017.01.014 (2017).
579 Rothers, J. *et al.* Maternal cytokine profiles during pregnancy predict asthma in children of nonasthmatic mothers. *Am J Respir Cell Mol Biol*, doi:10.1165/rcmb.2017-0410OC (2018).
580 Massey, C. A. *et al.* Isoflurane abolishes spontaneous firing of serotonin neurons and masks their pH/CO(2) chemosensitivity. *J Neurophysiol* **113**, 2879-2888, doi:10.1152/jn.01073.2014 (2015).
581 Richgels, P. K., Yamani, A., Chougnet, C. A. & Lewkowich, I. P. Maternal house dust mite exposure during pregnancy enhances severity of house dust mite-induced asthma in murine offspring. *J Allergy Clin Immunol*, doi:10.1016/j.jaci.2016.12.972 (2017).
582 Patel, K. R. *et al.* Mast cell-derived neurotrophin 4 mediates allergen-induced airway hyperinnervation in early life. *Mucosal Immunol* **9**, 1466-1476, doi:10.1038/mi.2016.11 (2016).
583 Johnson, J. R. *et al.* Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* **169**, 378-385, doi:10.1164/rccm.200308-1094OC (2004).
584 Tolloczko, B., Jia, Y. L. & Martin, J. G. Serotonin-evoked calcium transients in airway smooth muscle cells. *Am J Physiol* **269**, L234-240, doi:10.1152/ajplung.1995.269.2.L234 (1995).

585 Talbot, S. *et al.* Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation. *Neuron* **87**, 341-354, doi:10.1016/j.neuron.2015.06.007 (2015).
586 Fortner, C. N., Breyer, R. M. & Paul, R. J. EP2 receptors mediate airway relaxation to substance P, ATP, and PGE2. *Am J Physiol Lung Cell Mol Physiol* **281**, L469-474, doi:10.1152/ajplung.2001.281.2.L469 (2001).
587 Manzini, S. Bronchodilatation by tachykinins and capsaicin in the mouse main bronchus. *Br J Pharmacol* **105**, 968-972 (1992).
588 Kotas, M. E. & Medzhitov, R. Homeostasis, inflammation, and disease susceptibility. *Cell* **160**, 816-827, doi:10.1016/j.cell.2015.02.010 (2015).
589 Hotamisligil, G. S. Inflammation, metaflammation and immunometabolic disorders. *Nature* **542**, 177-185, doi:10.1038/nature21363 (2017).
590 Xu, H. *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* **112**, 1821-1830, doi:10.1172/JCI19451 (2003).
591 Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* **259**, 87-91 (1993).
592 McMaster, M. T., Newton, R. C., Dey, S. K. & Andrews, G. K. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *J Immunol* **148**, 1699-1705 (1992).
593 Robertson, S. A., Mau, V. J., Young, I. G. & Matthaei, K. I. Uterine eosinophils and reproductive performance in interleukin 5-deficient mice. *J Reprod Fertil* **120**, 423-432 (2000).
594 Eriksson, J. G., Kajantie, E., Osmond, C., Thornburg, K. & Barker, D. J. Boys live dangerously in the womb. *Am J Hum Biol* **22**, 330-335, doi:10.1002/ajhb.20995 (2010).
595 Osei-Kumah, A., Smith, R., Jurisica, I., Caniggia, I. & Clifton, V. L. Sex-specific differences in placental global gene expression in pregnancies complicated by asthma. *Placenta* **32**, 570-578, doi:10.1016/j.placenta.2011.05.005 (2011).
596 Chow, S. S. *et al.* Differences in amniotic fluid and maternal serum cytokine levels in early midtrimester women without evidence of infection. *Cytokine* **44**, 78-84, doi:10.1016/j.cyto.2008.06.009 (2008).
597 Moriyama, S. *et al.* beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. *Science* **359**, 1056-1061, doi:10.1126/science.aan4829 (2018).
598 Veres, T. Z. *et al.* Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation. *Am J Respir Cell Mol Biol* **37**, 553-561, doi:10.1165/rcmb.2007-0087OC (2007).
599 Wallrapp, A. *et al.* The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. *Nature* **549**, 351-356, doi:10.1038/nature24029 (2017).
600 Haldar, P. *et al.* Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* **360**, 973-984, doi:10.1056/NEJMoa0808991 (2009).
601 Martinez, F. D. Early-Life Origins of Chronic Obstructive Pulmonary Disease. *N Engl J Med* **375**, 871-878, doi:10.1056/NEJMra1603287 (2016).
602 Chiang, C. H. & Gabella, G. Quantitative study of the ganglion neurons of the mouse trachea. *Cell Tissue Res* **246**, 243-252 (1986).
603 Hardebo, J. E., Suzuki, N., Ekblad, E. & Owman, C. Vasoactive intestinal polypeptide and acetylcholine coexist with neuropeptide Y, dopamine-beta-hydroxylase, tyrosine hydroxylase, substance P or calcitonin gene-related peptide in neuronal subpopulations in cranial parasympathetic ganglia of rat. *Cell Tissue Res* **267**, 291-300 (1992).

604 Hoard, J. L. *et al.* Cholinergic neurons of mouse intrinsic cardiac ganglia contain noradrenergic enzymes, norepinephrine transporters, and the neurotrophin receptors tropomyosin-related kinase A and p75. *Neuroscience* **156**, 129-142, doi:10.1016/j.neuroscience.2008.06.063 (2008).
605 Leblanc, G. G. & Landis, S. C. Differentiation of noradrenergic traits in the principal neurons and small intensely fluorescent cells of the parasympathetic sphenopalatine ganglion of the rat. *Dev Biol* **131**, 44-59 (1989).
606 Springall, D. R. *et al.* Persistence of intrinsic neurones and possible phenotypic changes after extrinsic denervation of human respiratory tract by heart-lung transplantation. *Am Rev Respir Dis* **141**, 1538-1546, doi:10.1164/ajrccm/141.6.1538 (1990).
607 Zhou, Y. & Ling, E. A. Colocalization of nitric oxide synthase and some neurotransmitters in the intramural ganglia of the guinea pig urinary bladder. *J Comp Neurol* **394**, 496-505 (1998).
608 Yan, H. & Keast, J. R. Neurturin regulates postnatal differentiation of parasympathetic pelvic ganglion neurons, initial axonal projections, and maintenance of terminal fields in male urogenital organs. *J Comp Neurol* **507**, 1169-1183, doi:10.1002/cne.21593 (2008).
609 Kingham, P. J. *et al.* Effects of eosinophils on nerve cell morphology and development: the role of reactive oxygen species and p38 MAP kinase. *Am J Physiol Lung Cell Mol Physiol* **285**, L915-924, doi:10.1152/ajplung.00094.2003 (2003).
610 Gosens, R. *et al.* Muscarinic M(3) receptor-dependent regulation of airway smooth muscle contractile phenotype. *Br J Pharmacol* **141**, 943-950, doi:10.1038/sj.bjp.0705709 (2004).
611 Fauquet, M. & Ziller, C. A monoclonal antibody directed against quail tyrosine hydroxylase: description and use in immunocytochemical studies on differentiating neural crest cells. *J Histochem Cytochem* **37**, 1197-1205, doi:10.1177/37.8.2569003 (1989).
612 Leblanc, G. G. Coexpression of sensory and autonomic neurotransmitter traits by avian neural crest cells in vitro. *J Neurobiol* **21**, 567-577, doi:10.1002/neu.480210405 (1990).
613 Matsumoto, S. G. Neuronal differentiation in cultures of murine neural crest. II. Development of capsaicin-sensitive neurons. *Brain Res Dev Brain Res* **83**, 17-27 (1994).
614 Blaugrund, E. *et al.* Distinct subpopulations of enteric neuronal progenitors defined by time of development, sympathoadrenal lineage markers and Mash-1-dependence. *Development* **122**, 309-320 (1996).
615 Steinert, J. R. *et al.* Nitric oxide is an activity-dependent regulator of target neuron intrinsic excitability. *Neuron* **71**, 291-305, doi:10.1016/j.neuron.2011.05.037 (2011).
616 Anitha, M. *et al.* BMP2 promotes differentiation of nitrergic and catecholaminergic enteric neurons through a Smad1-dependent pathway. *Am J Physiol Gastrointest Liver Physiol* **298**, G375-383, doi:10.1152/ajpgi.00343.2009 (2010).
617 Hagendorens, M. M. *et al.* Prenatal exposure to house dust mite allergen (Der p 1), cord blood T cell phenotype and cytokine production and atopic dermatitis during the first year of life. *Pediatr Allergy Immunol* **15**, 308-315, doi:10.1111/j.1399-3038.2004.00169.x (2004).
618 LaMotte, R. H., Dong, X. & Ringkamp, M. Sensory neurons and circuits mediating itch. *Nat Rev Neurosci* **15**, 19-31, doi:10.1038/nrn3641 (2014).

619 Smyth, C. M. *et al.* Activated eosinophils in association with enteric nerves in inflammatory bowel disease. *PLoS One* **8**, e64216, doi:10.1371/journal.pone.0064216 (2013).

620 O'Brien, L. M., Fitzpatrick, E., Baird, A. W. & Campion, D. P. Eosinophil-nerve interactions and neuronal plasticity in rat gut associated lymphoid tissue (GALT) in response to enteric parasitism. *J Neuroimmunol* **197**, 1-9, doi:10.1016/j.jneuroim.2008.04.002 (2008).

621 Liu, Z. *et al.* Allergen challenge sensitizes TRPA1 in vagal sensory neurons and afferent C-fiber subtypes in guinea pig esophagus. *Am J Physiol Gastrointest Liver Physiol* **308**, G482-488, doi:10.1152/ajpgi.00374.2014 (2015).