The role of the ion channel TRPV4 in the Late Asthmatic Response

Pauline Léa Marie Flajolet

2021

A thesis submitted for the Degree of Doctor of Philosophy

in the Faculty of Medicine, Imperial College London

Respiratory Pharmacology Group
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Sir Alexander Fleming Building
Exhibition Road
London
SW7 2AZ

Abstract

In some patients, asthma is thought to be an atopic disease whereby exposure to allergens provokes symptoms. In fact, allergen inhalation can lead to a prolonged episode of airway narrowing named the late asthmatic response (LAR). Although it is a relevant clinical endpoint, the mechanisms driving it remain unclear. Evidence suggests that nerves could be involved, with allergen challenge resulting in the activation of airway sensory nerves within the vagus nerve and a subsequent reflex bronchospasm. This thesis investigated if the TRPV4 ion channel, a known activator of sensory nerves, could be a driver of the LAR.

The ability of TRPV4 to stimulate airway sensory nerves was studied in Brown Norway rats. A TRPV4 agonist depolarised rat vagal nerves and this was inhibited by TRPV4 and P2X3-P2X2/3 antagonists, suggesting that TRPV4 signalling induces the release of ATP which stimulates P2X3-P2X2/3 receptors on the neurons. Therefore, further studies interrogated whether this TRPV4-P2X3 nerve axis could be driving the LAR. Accordingly, both TRPV4 and P2X3-P2X2/3 antagonists inhibited the LAR induced by ovalbumin (OVA) in Brown Norway rats, suggesting a role for the TRPV4-P2X3 axis in experimental LAR. Seeing these results, the mechanism leading to TRPV4 activation was investigated. PAR2 receptors, which are activated by proteases released upon allergen challenge, were considered as promising TRPV4 activators. Indeed, a PAR2 agonist depolarised rat vagal nerves and this was blocked by PAR2, TRPV4 and P2X3-P2X2/3 antagonists. However, two PAR2 antagonists did not reduce the LAR in the rat OVA model, suggesting that PAR2 receptors may not be involved in this model.

Overall, these data suggest that the TRPV4-P2X3 axis could play a role in the LAR by activating airway sensory nerves. This thesis highlights that investigating receptors on airway sensory nerves could help to develop asthma therapies targeting alternative mechanisms than immune pathways.

Acknowledgements

I would like to thank my supervisors Dr Mark Birrell and Prof Maria Belvisi for providing me with the opportunity to complete my PhD within the Respiratory Pharmacology group. I am extremely grateful for your support, guidance and scientific expertise, not only throughout my PhD but also throughout all the previous internships spent in your group.

I would like to thank the NHLI for funding my PhD studentship. My thanks also go to Dr Ranganath Gopalakrishnan and Dr Werngard Czechtizky from AstraZeneca for the provision of the PAR2 antagonist compounds. I would also like to thank Stephen Rothery from the FILM Facility at Imperial College London for helping me with the analysis of PCLS imaging.

A very special thanks to my unofficial supervisor, Dr Sara Bonvini, for countless hours of guidance, support, vagus experiments, proofreading and also for trusting me with writing projects. Special thanks also to my mentor, Dr John Adcock, for providing invaluable scientific and emotional support throughout this PhD adventure, always with a twist of 70's, great stories and a good sense of humour.

A big thanks to the Respiratory Pharmacology group, without whom this PhD would not exist. Thank you to Bilel Dekkak (for your help during all these years and always making me laugh), Dr Eric Dubuis (for being such a kind genius and saving my experiments countless times), Dr Nadja Kobold (my twin lab buddy), Shirley Chen (my work wife), Dr Thomais Markou and Dimitrios Zervas (for all your hard work and bravery with PCLS), Dr James Pinkerton (for showing me that studies can be enjoyable), Jonathan Miles (for your kindness and sharing my crazy thoughts), Dr Michael Wortley (for your expertise and dark humour) and Dr Vincent Cibert-Goton (for helping me with etrec).

I would especially like to thank Nadja, Shirley and Thomais for your friendship and support. To Nadja, for being my partner in crime and teaching me what really matters in life. To Shirley, for always being there and keeping me entertained (and fed) at all times. To Thomais, for your incredible kindness and for laughing at my French jokes.

I would also like to thank my friends, Virginie, Marie, Lorph, Lane and Awen, for your incredible support even from across the border. Thank you to "la guilde des zinzins", Jordan, Léo, Mika, for keeping me sane during these last months of writing. Thank you to les Garniers for putting up with me during all these years. To my parents, thank you for your unconditional support and encouragement during this 10-year long journey. Last but not least, all my love and thanks to Anthony, for always supporting me, I cannot wait to start this next chapter with you.

Statement of Originality

This thesis is the product of my own work, except where appropriately referenced.

Isolated vagus experiments in Chapters 3 and 6 were performed by Dr Sara Bonvini. The isolated tracheal nerve experiment in Chapter 3 was performed by Dr Eric Dubuis with my assistance. Single fibre experiments in Chapters 3 and 6 were performed by Dr John Adcock with my assistance. The genotyping of *Par2*$^{-/-}$ mice in Chapter 6 was performed by Dr Nadja Kobold. Isolated vagus experiments in Chapter 7 were performed by Dr Michael Wortley.

This PhD project was funded by the NHLI Foundation studentship.

Copyright Declaration

Table of contents

List of Figures

List of Tables

List of Abbreviations

2f-LIGRLO-NH2	2-furoyl-LIGRLO-amide
4αPDD	4α-Phorbol 12,13-didecanoate
5HT	Serotonin
5-HTR	Serotonin Receptor
αβ-MeATP	αβ-Methylene ATP
ACh	Acetylcholine
AHR	Airway Hyperresponsiveness
APC	Antigen Presenting Cell
ASM	Airway Smooth Muscle
AMP, ADP, ATP	Adenosine Monophosphate, Diphosphate, Triphosphate
ATPase	Adenosine Triphosphatase
AUC	Area Under Curve
BAL	Bronchoalveolar Lavage
CA	Citric Acid
CCh	Carbachol
CCL17, CCL22	Chemokine (C-C motif) ligand 17, 22
CCR3	C-C Motif Chemokine Receptor 3
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CRTH2	Chemoattractant receptor-homologous molecule expressed on T_H2 cells
Cryo-EM	Cryogenic Electron Microscopy
CYPe	Cytochrome epoxygenase pathway
CysLT	Cysteinyl Leukotriene
CysLTR1	Cysteinyl Leukotriene receptor 1
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's medium
DMN	Dorsal Motor Nucleus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal Root Ganglia
EAR	Early Asthmatic Response
EB	Eosinophilic Bronchitis
ECS	Extracellular Solution
EET	Epoxyeicosatrienoic acid
EFS	Electrical Field Stimulation

EtOH	Ethanol
FBS	Fetal Bovine Serum
FcERI	Fc-epsilon receptor I
FEV1	Forced Expiratory Volume in one second
FWBP	Flow Whole Body Plethysmography
GATA3	Globin transcription factor Binding Protein 3
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G Protein Coupled Receptor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H1R	Histamine H1 receptor
HBSS	Hanks' Balanced Salt Solution
HDM	House Dust Mite
HETE	Hydroxyeicosatetraenoic acid
HLA	Human Leukocyte Antigen
HPETE	Hydroperoxyeicosatetranenoic acid
HP-β-cyclodextrin	6% (2-Hydroxypropyl)-β-cyclodextrin
ICS	Inhaled Corticosteroids
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-4, 5, 13	Interleukin 4, 5, 13
ILC2	Innate Lymphoid Cells type 2
i.n.	Intranasal
i.p.	Intraperitoneal
IP_3	Inositol 1,4,5-trisphosphate
i.t.	Intratracheal
i.v.	Intravascular
JUG	Jugular ganglia
KH	Krebs-Henseleit solution
K_v	Potassium Voltage Gated Channels
LABA	Long-Acting β2-adrenoceptor Agonist
LAMA	Long-Acting Muscarinic receptor Antagonist
LAR	Late Asthmatic Response
LDH	Lactate dehydrogenase
LTC_4, D_4, E_4	Leukotriene C4, D4, E4
LTRA	Leukotriene Receptor Antagonist
M1, 2, 3	Muscarinic receptor 1, 2, 3
MCh	Methacholine
MEM	Minimum Essential Media

MHCII	Major Histocompatibility Complex class II
MMM	Montelukast Mepyramine Methysergide
NA	Nucleus Ambiguus
NANC	Non-Adrenergic Non-Cholinergic
Na_v	Voltage Gated Sodium Channels
NKA	Neurokinin A
NMA	Nerve Mass Activity
NO	Nitric Oxide
NOD	Nodose Ganglia
dNTP	Deoxynucleoside triphosphate
NTS	Nucleus Tractus Solitarii
OVA	Ovalbumin
P2X, P2Y	Purinergic receptor type 2 isoform, X and Y
Panx1	Pannexin 1 channel
PAR1, 2	Protease-Activated Receptor 1, 2
PCLS	Precision-Cut Lung Slices
PCR	Polymerase Chain Reaction
PEG400	Polyethylene glycol 400
Penh	Enhanced pause
PGD_2, PGE_2	Prostaglandin D2, E2
PKA, PKC, PKD	Protein Kinase A, C, D
PLA2, PLC	Phospholipase A2, Phospholipase C
pNA	p-Nitroanilide
p.o.	per os (oral)
PRR	Pattern Recognition Receptor
PΣ	Parasympathetic
RAR	Rapidly Adapting Receptor
RLN	Recurrent Laryngeal Nerve
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
SABA	Short Acting β2-adrenoceptor Agonist
SAL	Saline
SAR	Slowly Adapting Receptor
s.c.	Subcutaneous
Scr	Scrambled peptide
SEM	Standard Error of the Mean
SLN	Superior Laryngeal Nerve

TCR	T Cell Receptor
TGFβ	Transforming Growth Factor β
Th cells	T helper cells
Treg	Regulatory T cells
TRP	Transient Receptor Potential
TSLP	Thymic Stromal Lymphopoietin
TTX	Tetrodotoxin
VCAM-1	Vascular Adhesion Molecule I
Veh	Vehicle
VIP	Vasoactive Intestinal Peptide
VRG	Ventral Respiratory Group
w/v	weight/volume

1 Introduction

1.1 Asthma

1.1.1 Definition of asthma

Asthma is a major public health burden, with a reported global prevalence of 272 million asthmatics in 2017 (James et al. 2018). Asthma has been defined as a chronic inflammatory disease characterised by recurrent episodes of wheezing, shortness of breath, chest tightness and cough as well as airway hyperresponsiveness (AHR), which is an excessive bronchospasm triggered by nonspecific stimuli. However, recent developments have revealed that the label “asthma” encompasses many different pathological processes (endotypes) leading to many different manifestations (phenotypes). Therefore, asthma is now recognised as a syndrome, with two key features: a history of respiratory symptoms and the presence of variable expiratory airflow limitation (Pavord et al. 2018; Global Initiative for Asthma 2019).

1.1.2 Asthma heterogeneity

Clinical clusters of asthma have been delineated according to age of onset, atopic status, pattern and severity of respiratory symptoms and inflammation (Gauthier, Ray, and Wenzel 2015). Among them, atopic asthma is the most commonly encountered form, usually with an early-onset in childhood. Patients with atopic asthma produce IgE specific to innocuous antigens called allergens, attested by the presence of specific IgE in serum or a positive skin prick test (Comberiati et al. 2017). In these patients, inhalation of a specific allergen, most commonly house dust mite (HDM), pollen, animal dander or fungal spores, is thought to actively participate in the pathophysiology (Gauvreau, El-Gammal, and O’Byrne 2015).

Moving away from clinical phenotyping, research has recently focused on defining subtypes of asthma according to the molecular pathways involved. Asthma has been originally considered as a prototypical disease of type-2 adaptive immunity, whereby T helper 2 (T_H2) cells drive airway inflammation by secreting the type-2 cytokines interleukins 4, 5 and 13 (IL-4, IL-5, IL-13), leading to airway eosinophilia, production of allergen-specific IgE, AHR, mucus hypersecretion and airway remodelling. Asthma presentations associated with this eosinophilic inflammation have been labelled as “T_H2 high”. However, recent studies have revealed that innate cells such as group 2 innate lymphoid cells (ILC2s), basophils and mast cells could also be responsible for producing type-2 cytokines and driving the pathology, therefore the label “T_H2 high” was replaced by “Type-2 high”. In contrast, an important proportion of patients do not show evidence of type-2 inflammation, with a wide spectrum of

presentations ranging from neutrophilia to pauci-granulocytic inflammation which are currently considered as “Type-2 low” asthma (Gauthier, Ray, and Wenzel 2015; Lambrecht, Hammad, and Fahy 2019). Therefore, with the advance of “omics” technologies, asthma unravelled itself as a label regrouping heterogeneous processes and this classification is likely to evolve, with the current aim to identify molecular pathways responsible for a given phenotype in a specific patient to deliver precision medicine (Pavord et al. 2018).

1.1.3 Current asthma therapies

Asthma management currently aims to reduce two traits: the occurrence of symptoms as a result of variable expiratory airflow limitation and the risk of asthma attacks (Pavord et al. 2018).

Airflow limitation has been attributed to excessive airway smooth muscle contraction either to specific (allergens) or non-specific (exercise, cold air) stimuli, but also to mucus hypersecretion, oedema and airway remodelling when persistent (Barnes 2017). Bronchodilators are used to relieve and prevent symptoms due to airflow limitation, with the gold standard long-acting β2-adrenoreceptor agonists (LABA) formoterol and salmeterol. With a different mechanism of action, leukotrienes receptor antagonists (LTRA) (e.g. montelukast) and the long-acting muscarinic antagonist (LAMA) tiotropium are also used as add-on bronchodilators (Global Initiative for Asthma 2019).

On the other hand, airway inflammation has been linked with the risk of asthma attacks, which are acute episodes of increased symptoms with a high risk of mortality and increased disease progression (Pavord et al. 2018). Therefore, anti-inflammatory therapies are a cornerstone of asthma management, with the gold standard anti-inflammatory inhaled corticosteroids (ICS) (e.g. beclomethasone, budesonide) (Global Initiative for Asthma 2019). New biological therapies targeting specific inflammatory pathways are also emerging and aimed at specific subgroups of patients. The anti-IgE monoclonal antibody omalizumab has been approved as add-on therapy in atopic asthma and four monoclonal antibodies targeting type-2 cytokines as add-on therapies in severe asthma associated with a type-2 high phenotype, namely mepolizumab (anti-IL-5), reslizumab (anti-IL-5), benralizumab (anti-IL-5Rα) and dupilumab (anti-IL-4Rα) (Global Initiative for Asthma 2019).

Current guidelines recommend that asthma management relies on the combination of anti-inflammatory and bronchodilator therapies as a controller medication to prevent attacks and symptoms, with ICS-LABA being the mainstay of controller therapy. This approach is conducted in a step-wise manner, by increasing ICS-LABA doses and/or adding complementary therapies (LTRA, LAMA, biologicals) until satisfactory control is achieved

(**Figure 1-1**). As of 2019, ICS-LABA is also used as a reliever medication taken in case of symptoms occurring in spite of the controller therapy (Global Initiative for Asthma 2019).

	STEP 1	**STEP 2**	**STEP 3**	**STEP** 4	***STEP 5***
Preferred controller	As needed low dose ICS-formoterol	Daily low dose ICS or as-needed low dose ICS-formoterol	Daily low dose ICS-LABA	Daily medium dose ICS-LABA	Daily high dose ICS-LABA ± add-on tiotropium anti-IgE anti-IL-5/IL-5R anti-IL-4R
Other options	*Low dose ICS whenever SABA taken*	*LTRA or low dose ICS whenever SABA taken*	*Medium dose ICS or low dose ICS + LTRA*	*High dose ICS ± tiotropium or LTRA*	*Add low dose OCS*
Preferred reliever	As needed low dose ICS-formoterol				
Other option	*As needed SABA*				

Figure 1-1 GINA guidelines 2019 for asthma management in asthmatics 12+ years
Adapted from (Global Initiative for Asthma 2019). ICS: Inhaled Corticosteroid, SABA: Short-Acting β2-adrenoreceptor Agonist, LTRA: Leukotrienes Receptor Antagonist, LABA: Long-Acting β2-adrenoreceptor Agonist.

Despite current therapies, an important number of patients remain uncontrolled, with either poor symptom control, acute exacerbations and/or presenting airflow limitation (Chung et al. 2014). In 2014, an online survey of 8000 European asthmatic patients found that overall 45% of the respondents had uncontrolled asthma, ranging from 29 to 49% across GINA-defined treatment levels (Price, Fletcher, and van der Molen 2014). In addition of the burden of experiencing symptoms, uncontrolled asthma has major consequences as it is a high risk factor for asthma attacks and therefore a high risk of mortality (Price, Fletcher, and van der Molen 2014; Pavord et al. 2018). In addition, an estimated 5 to 10% asthmatic patients have severe asthma that requires a high dose of corticosteroids or remains uncontrolled despite this therapy (Chung et al. 2014). While uncontrolled asthma may be due to non-adherence, a significant proportion of patient does not respond to current therapies. Therefore, it is necessary to decipher different molecular pathways than those targeted by current therapies. This thesis will be focused on investigating new molecular pathways involved in provoking airflow limitation in atopic asthma.

1.2 Atopic asthma pathophysiology

Atopic asthma has been considered as the result of a dysregulated type-2 adaptive response to an innocuous antigen driven by T_H2 cells, with the presence of IgE and eosinophilia being hallmarks of T_H2 inflammation. However, recent findings have suggested that innate immune cells also play an important role in driving type-2 responses. This section summarises the current paradigm on how airway inflammation develops in atopic asthma, with the nuance that pathophysiological processes are likely to differ between patients (Gauthier, Ray, and Wenzel 2015; Lambrecht, Hammad, and Fahy 2019).

1.2.1 Adaptive type-2 immunity in atopic asthma

As previously mentioned, the traditional paradigm views atopic asthma as resulting from an aberrant type-2 adaptive response to an innocuous antigen named allergen, driven by T_H2 cells. In this paradigm, T_H2-driven airway inflammation involves two phases: sensitisation upon initial allergen inhalation and allergic airway response upon subsequent allergen re-exposure (Lambrecht and Hammad 2015; Lambrecht, Hammad, and Fahy 2019; Lloyd and Snelgrove 2018).

1.2.1.1 Sensitisation to the allergen

In atopic patients, initial exposure to an allergen can lead to sensitisation, that is the priming of immune cells to induce allergic responses to the specific allergen. Upon initial exposure, the inhaled allergen is captured and processed by dendritic cells (DCs) in the airways. DCs migrate to lung draining lymph nodes and present processed allergen peptides via major histocompatibility complex class II (MHC II) molecules to naïve $CD4^+$ T helper cells (T_H0) bearing T cell receptors (TCRs) specific to the allergen. This triggers the differentiation of T_H0 cells into allergen-specific T_H2 cells producing the type-2 cytokines IL-4, IL-5 and IL-13 under the master transcription factor GATA3. Following DC-T_H2 interaction, subsets of T_H2 cells migrate to B cell zones of the lymph node and differentiate into T follicular helper cells (T_{FH}) that interact with allergen-specific B cells and secrete IL-4, inducing immunoglobulin class switching and the production of allergen-specific IgE by B cells. Other T_H2 cells migrate to the lung tissue and produce IL-5 and IL-13. Circulating IgE binds to high affinity receptors FcεRI expressed on tissue-resident mast cells and circulating basophils but also on other innate cells such as DCs, priming these immune cells to respond to future allergen exposure and elicit type-2 responses (Lambrecht and Hammad 2015; Lambrecht, Hammad, and Fahy 2019; Lloyd and Snelgrove 2018).

1.2.1.2 Re-exposure to the allergen

Upon re-exposure, the allergen crosslinks IgE-FcεRI complexes on tissue-resident mast cells and recruited basophils, leading to the release of mediators such as cysteinyl-leukotrienes (CysLTs) which are potent inducers of bronchoconstriction, airway oedema and mucus secretion. In addition, allergen exposure activates local monocyte-derived cells such as DCs and macrophages, that recruit circulating T_H2 cells in the airways via secreting chemokines such as CCL17, CCL22 and reactivate T_H2 cells via IgE-dependent allergen presentation. T_H2 effector cells thereby produce IL-4, IL-5 and IL-13 cytokines which promote type-2 responses (**Figure 1-2**). Mast cells and basophils activated by IgE-FcεRI crosslinking also produce IL-4 and IL-13, amplifying the reaction (Lambrecht and Hammad 2015; Lambrecht, Hammad, and Fahy 2019).

Based on observations originally made in mouse models of allergic airway disease induced by ovalbumin (OVA), these cytokines IL-4, IL-5 and IL-13 are thought to be responsible for orchestrating the pathophysiological changes seen in atopic asthma, that are airway eosinophilia, AHR, excessive mucus production and airway remodelling (**Figure 1-2**) (Lambrecht and Hammad 2015; Lambrecht, Hammad, and Fahy 2019).

1.2.1.3 Effects of IL-5 cytokine

Indeed, mouse models have demonstrated that IL-5 has a critical role in inducing airway eosinophilia, by promoting eosinophil expansion and differentiation in the bone marrow and the release of eosinophils into the circulation. Chemokines named eotaxins secreted in inflamed airways bind to CCR3 receptors on circulating eosinophils and induce their recruitment to the airways. In airway tissues, IL-5 promotes the survival of recruited eosinophils (Lambrecht, Persson, and Hammad 2017). The crucial role of IL-5 in inducing eosinophilia has been confirmed in human with the testing of the anti-IL-5 monoclonal antibodies mepolizumab (anti-IL-5), reslizumab (anti-IL-5) and especially benralizumab (anti-IL-5Rα) which profoundly reduce blood and tissue eosinophil counts in patients with severe uncontrolled asthma and high blood eosinophilia (Haldar et al. 2009; Bjermer et al. 2016; Castro et al. 2015; Bleecker et al. 2016; FitzGerald et al. 2016). Although the precise role of eosinophilia in asthma remains unclear, clinical studies have suggested that it is linked to the risk of asthma exacerbations (Haldar et al. 2009; Castro et al. 2015; Bleecker et al. 2016; FitzGerald et al. 2016; Yancey et al. 2017).

1.2.1.4 Effects of IL-4 and IL-13 cytokines

IL-4 binds to IL-4Rα subunit, which can be found assembled as IL-4Rα/γC heterodimers on hematopoietic cells and IL-4Rα/IL-13Rα1 heterodimers on non-hematopoietic cells. IL-13 binds to IL-13Rα1 subunit and therefore shares with IL-4 the common receptor IL-4Rα/IL-13Rα1. As a result, it has been shown that IL-4 and IL-13 can provoke complementary and redundant actions, although IL-4 and IL-13 appear to be secreted at different locations and could therefore be involved in distinct processes (Marone et al. 2019; Lambrecht, Hammad, and Fahy 2019).

IL-4 is a prototypical T_H2 cytokine as it drives T_H0 differentiation into T_H2 cells and induces IgE production by B cell class switching from IgM (Steinke and Borish 2001). IL-13 can also induce the production of IgE, however IL-4 was shown to be mostly produced by T_{FH} cells that are located in lymph nodes and interact with B cells (Marone et al. 2019; Lambrecht, Hammad, and Fahy 2019).

IL-4 and IL-13 have also been shown to promote airway eosinophilia. Indeed, IL-4 and IL-13 upregulate vascular cell adhesion molecule-I (VCAM-I) on endothelial cells, allowing the endothelial transmigration of circulating eosinophils into the airways. Anti-IL-4 and anti-IL-13 therapies have indeed been associated with elevated eosinophil counts in the blood but a decrease in the airway tissue compartment. In addition, IL-4 and IL-13 promote eosinophil chemotaxis via driving the production of eotaxins by epithelial cells (Steinke and Borish 2001; Marone et al. 2019; Lambrecht, Persson, and Hammad 2017).

In mouse models, IL-13 is thought to promote goblet cell metaplasia, subepithelial fibrosis and AHR, although the link between IL-13 and AHR is controversial (Grünig et al. 1998; Kumar et al. 2002; Webb et al. 2000; Cohn, Tepper, and Bottomly 1998). Studies have also shown that IL-4 exerts redundant effects (Grünig et al. 1998; Perkins, Wills-Karp, and Finkelman 2006; Brusselle et al. 1994). As IL-4 and IL-13 share a common receptor, it has been hypothesised that IL-13 may be more important in exerting these effects because of a higher production of IL-13 at effector sites (Lambrecht 2019, Perkins 2006). Nonetheless, anti-IL-13 and anti-IL-4 monoclonal antibodies have showed inconsistent results in asthmatic patients and therefore their biological effects remain uncertain (Marone et al. 2019). Efficacy was however obtained in severe asthma with the anti-IL-4Rα monoclonal antibody dupilumab on reducing asthma exacerbations. Dupilumab inhibits both IL-4 and IL-13 signalling, perhaps reflecting the redundancy of IL-4 and IL-13 actions (S. Wenzel et al. 2016; Castro et al. 2018; Rabe et al. 2018).

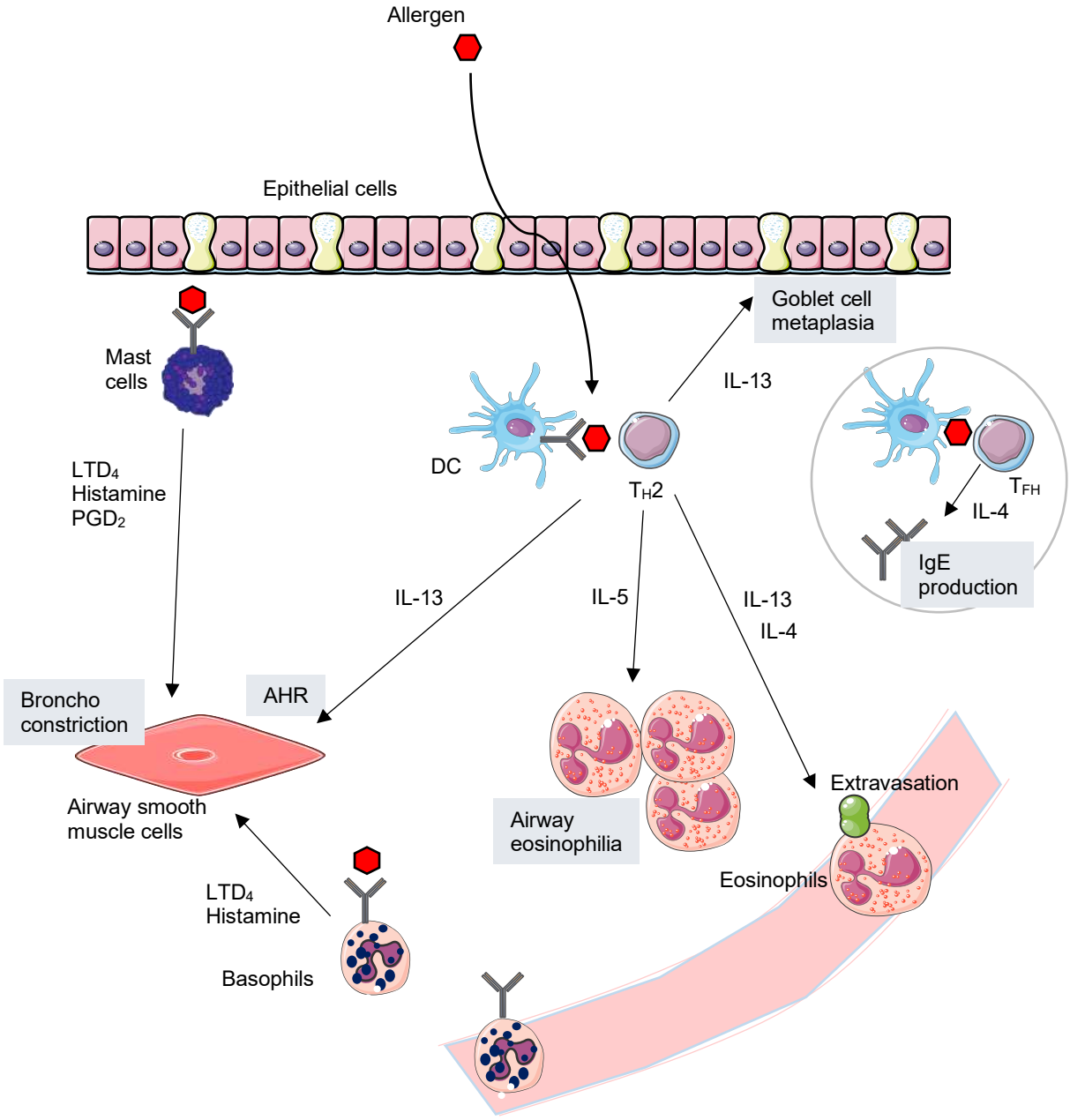


Figure 1-2 T_H2-driven airway inflammation upon allergen challenge

Representation of simplified inflammatory events triggered by allergen challenge and leading to T_H2-driven airway eosinophilia, AHR and goblet cell metaplasia. In sensitised individuals, allergen challenge leads to the activation of T_H2 subsets that produce IL-4, IL-5 and IL-13. In lymph nodes, the secretion of IL-4 by T_{FH} induces the production of specific IgE by B cells. Mast cells and basophils armed with specific IgE degranulate upon allergen challenge and secrete a wide range of mediators provoking bronchoconstriction. In the airways, type-2 cytokines trigger airway eosinophilia: IL-13 induces the secretion of eotaxins that recruit eosinophils to the airways, IL-4 and IL-13 upregulate endothelial adhesion molecules that promote eosinophil extravasation and IL-5 promotes the survival of eosinophils in the tissues. IL-13 has also been involved in provoking goblet cell metaplasia and AHR. Adapted from (Lambrecht, Persson, and Hammad 2017; Lambrecht, Hammad, and Fahy 2019).

1.2.2 Innate type-2 immunity in atopic asthma

It remains unclear why certain patients would develop a type-2 adaptive response to allergens. Genetic variants and epigenetic regulation of genes encoding cytokines and receptors involved in the adaptive response have been associated with an increased risk of asthma (Lambrecht, Hammad, and Fahy 2019). Environmental factors are also increasingly depicted as important factors shaping type-2 adaptive responses, including microbiome composition, with the influence of hygiene, diet and infectious diseases, as well as exposure to pollutants. DCs appear to be crucial in determining the polarisation of the T cell response and the activation of pattern recognition receptors (PRRs) or G protein coupled receptors (GPCRs) by certain microbiome-derived metabolites have been shown to promote T_H2-polarising DCs (Lambrecht and Hammad 2017).

However, the recent development of mouse models of allergic airway disease using natural allergens such as house dust mite (HDM) have revealed that innate immunity could also play an important role in shaping type-2 adaptive responses. Indeed, allergens such as HDM, *Alternaria* fungi or papain can activate PRRs or protease-activated receptors 2 (PAR2) on epithelial cells and induce their rapid release of “alarmins” cytokines including IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) (Lambrecht, Hammad, and Fahy 2019; Hammad and Lambrecht 2015). These cytokines IL-33, IL-25 and TSLP were shown to activate a subset of DCs that promote T_H2 polarisation (Lambrecht, Hammad, and Fahy 2019; Hammad and Lambrecht 2015). In addition, studies have suggested that air pollutants and microbiome products can also activate PRRs on epithelial cells and modulate their release of alarmins, thereby influencing the adaptive immune response (Hammad and Lambrecht 2015).

Importantly, these mouse models led to the discovery of group 2 innate lymphoid cells (ILC2s) that are tissue-resident innate immune cells that lack antigen specific receptors but produce IL-5 and IL-13. Upon allergen exposure, epithelial-derived IL-33, IL-25 and TSLP activate ILC2s to release IL-5 and IL-13 (Lambrecht, Hammad, and Fahy 2019; Hammad and Lambrecht 2015) (**Figure 1-3**). In mouse models, ILC2s have been involved in the sensitisation to allergens, potentially by acting as APCs via their expression of MHC II and by secreting IL-13 that promote DCs migration to lymph nodes and the subsequent priming of T_H2 cells (Lambrecht, Hammad, and Fahy 2019; Lloyd and Snelgrove 2018; Halim et al. 2014). ILC2s have also been involved in the T_H2 effector response upon allergen re-challenge, by secreting IL-13 that stimulate CCL17 production by DCs and the recruitment of T_H2 cells to the airways (Halim et al. 2016).

In fact, these mouse models using natural allergens also revealed that other cells than T_H2 cells could drive type-2 responses, with studies suggesting that ILC2s secrete significant

amounts of IL-5 and IL-13 that can provoke eosinophilia, goblet cell metaplasia and AHR in the absence of B and T cells. In addition, mast cells and basophils also secrete IL-4 and IL-13 upon stimulation by IL-33, IL-25 and TSLP (Lambrecht, Hammad, and Fahy 2019).

These findings led to re-evaluate the paradigm of a central DC-T_H2 response in favour of an integrated response, where the local environment is perceived by tissue-resident cells that signal changes to innate immune cells. The latter can induce early type-2 responses and/or instruct adaptive immune cells to mount a specific type-2 response with a longer time span (Lloyd and Snelgrove 2018) (**Figure 1-3**).

It is however unclear how T_H2 and ILC2 responses are interdependent in atopic asthma, as their relative roles depend on the mouse model used (Halim et al. 2014; B. W. S. Li et al. 2016) and the contribution of ILC2s is yet to be determined in human atopic asthma. Nonetheless, it has been suggested that ILC2 cells could be more important in severe forms of eosinophilic inflammation found in older patients, that are less responsive to corticosteroids and associated with chronic rhinosinusitis with nasal polyposis (Lambrecht, Hammad, and Fahy 2019).

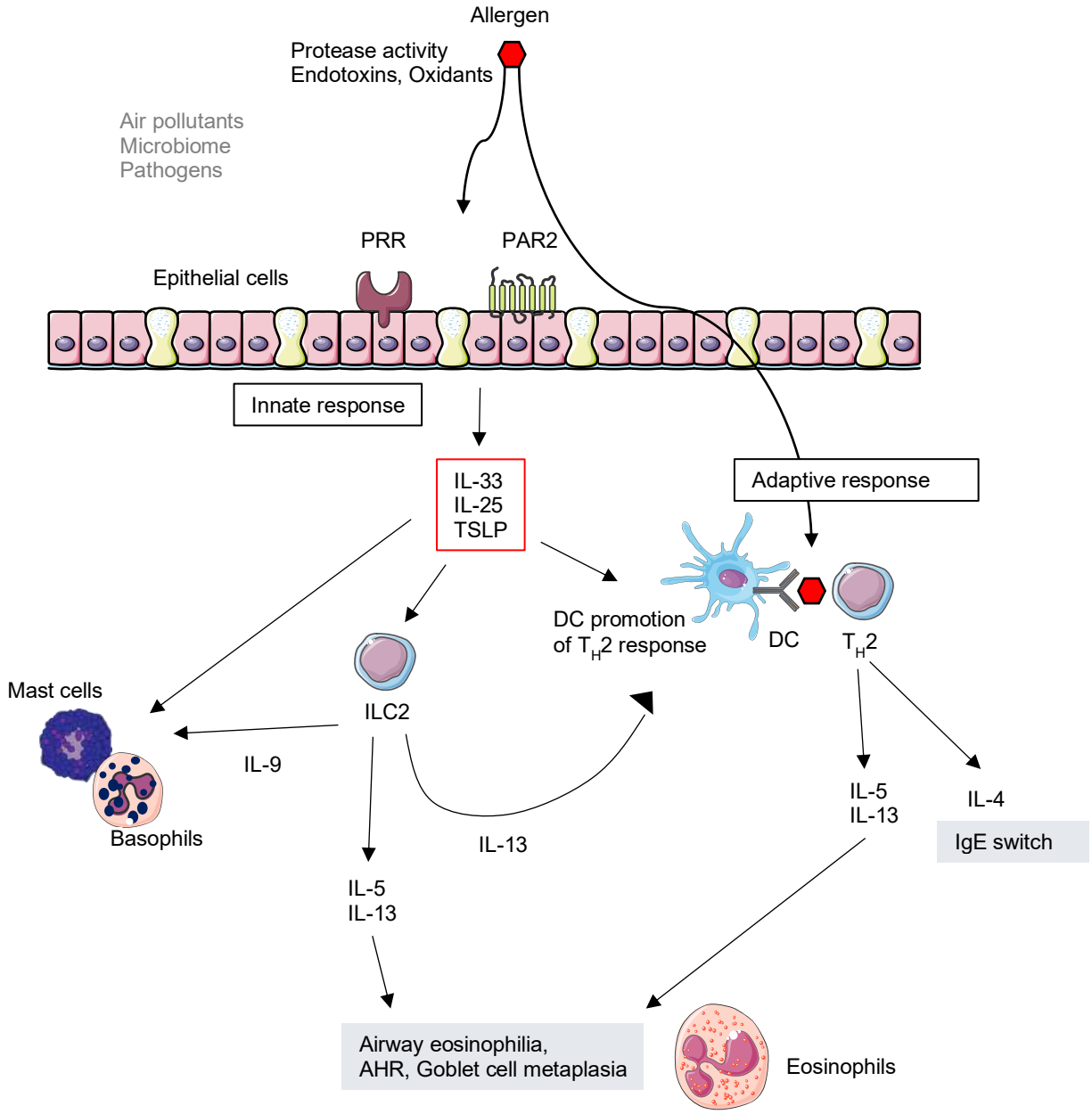


Figure 1-3 Interplay between innate and adaptive type-2 responses in asthma

Representation of simplified and hypothetical interactions between innate and adaptive immune cells in atopic asthma. Allergens can stimulate PRRs and PAR2 receptors on epithelial cells, potentially via their protease activity, endotoxin content or oxidant activity, thereby triggering the rapid release of alarmins IL-33, IL-25 and TSLP amongst other danger signals. Components of the environment such as air pollutants or microbiome-derived products can also modulate this epithelial response. Released alarmins stimulate innate cells such as ILC2s, mast cells and basophils to secrete the type-2 cytokines IL-4, IL-5 and IL-13 in varying abundance. Activated ILC2s produce significant amounts of IL-5 and IL-13, which can potentially induce type-2 responses independently from T_H2 cells but also boost DC-T cells interactions to mount a type-2 adaptive response specific to the allergen. In addition, ILC2s produce IL-9 that stimulates mast cells expansion and survival. Based on (Lloyd and Snelgrove 2018; Lambrecht, Hammad, and Fahy 2019).

1.3 Allergen inhalation challenge

As patients are continuously exposed to allergens, it is hard to decipher the different mechanisms involved in the pathology. However, in the laboratory, inhalation of a high dose of a specific allergen by mild stable atopic asthmatics replicates key features of asthma, with reversible airflow obstruction specific to the allergen, eosinophilic airway inflammation and AHR to nonspecific stimuli such as the bronchoconstrictor methacholine (MCh). Allergen inhalation challenge studies therefore provide a model to study the temporality and the mechanistic of events leading to airway inflammation and airflow limitation driven by allergic mechanisms in mild atopic asthmatics (Gauvreau, El-Gammal, and O'Byrne 2015; O'Byrne, Gauvreau, and Brannan 2009). This same principle is exploited in animal models of asthma, with animals being firstly sensitised to an allergen then exposed to allergen topical challenge with various frequencies. In fact, most of the mechanisms of airway inflammation depicted in previous sections comes from human and animal allergen challenge studies (Gauvreau, El-Gammal, and O'Byrne 2015; Lambrecht and Hammad 2015; Lambrecht, Hammad, and Fahy 2019).

Although allergen-induced bronchoconstriction has received less attention than airway inflammation in preclinical models, reversible airflow obstruction is a key feature of atopic asthma. In the laboratory, acute inhalation of a high dose of a specific allergen by mild stable atopic asthmatics provokes a decrease in lung function FEV_1 (≥ 20% from baseline) within 10 min and resolving within 2 h named the early asthmatic response (EAR). Around half of the patients also experience a delayed and prolonged decrease in FEV_1 (≥ 15 % from baseline) known as the late asthmatic response (LAR), occurring from 3 to 7 h and with a maximum between 8 and 12 h post-challenge (Diamant et al. 2013) (**Figure 1-4**). The LAR is characteristically accompanied by an influx of inflammatory cells in the airways that comprises mostly eosinophils but also basophils and neutrophils. This late response is also associated with the development of AHR, evaluated by a left-shift in the concentration-response to bronchoconstrictors such as methacholine (MCh) or histamine (Diamant et al. 2013).

1.3.1 The early asthmatic response (EAR)

It is agreed that the EAR results from the allergen crosslinking IgE-FcεRI complexes on tissue-resident mast cells and inducing their fast release of preformed histamine and *de novo* synthesised CysLTs LTC_4, LTD_4 and prostaglandin PGD_2, which induce airway smooth muscle contraction, mucus secretion and vascular leakage. In order of importance, CysLTs are thought to account for the majority of the EAR, followed by histamine and to a lesser extent PGD_2 (Gauvreau, El-Gammal, and O'Byrne 2015; Bradding and Arthur 2016), a finding supported by allergen challenge studies testing CysLTs receptor antagonists (LTRAs) and histamine H1 receptor antagonists on human EAR (Roquet et al. 1997; Hamilton et al. 1998; Leigh et al. 2002; Diamant et al. 1999; Davis et al. 2009).

1.3.2 The late asthmatic response (LAR)

The mechanism of the LAR is lesser known than the EAR and yet, the LAR is considered to be a more relevant endpoint for studying mechanisms of allergen-induced airflow limitation and inflammation in atopic asthma, for one fact that it is a prolonged drop in lung function and is associated with inflammatory cell influx and AHR (Gauvreau, El-Gammal, and O'Byrne 2015; O'Byrne, Gauvreau, and Brannan 2009). More importantly, a reduction of the LAR harbours important negative predictive value on the reduction of symptoms due to airflow limitation. As such, the gold standard therapies ICS, LABAs, LTRAs and anti-IgE omalizumab have all shown an important reduction of the LAR in atopic asthmatics (Gauvreau, El-Gammal, and O'Byrne 2015; O'Byrne, Gauvreau, and Brannan 2009).

The inhibition of the LAR by long-acting β2 agonists (LABAs) suggests that LAR entails bronchoconstriction (Dente et al. 1999; Pizzichini et al. 1996; Duong et al. 2007). Due to the potent reduction of the LAR and associated inflammation exerted by corticosteroids, the LAR is thought to result from the recruitment of eosinophils and basophils in the airways and releasing mediators such as CysLTs causing bronchoconstriction, mucus secretion and oedema (Gauvreau, El-Gammal, and O'Byrne 2015; O'Byrne, Gauvreau, and Brannan 2009). However, the causal link between airway inflammation and airflow limitation is still unclear. For this reason, this thesis aimed to investigate possible mechanisms driving the LAR, which could lead to identify new endotypes associated with airflow limitation in atopic asthma.

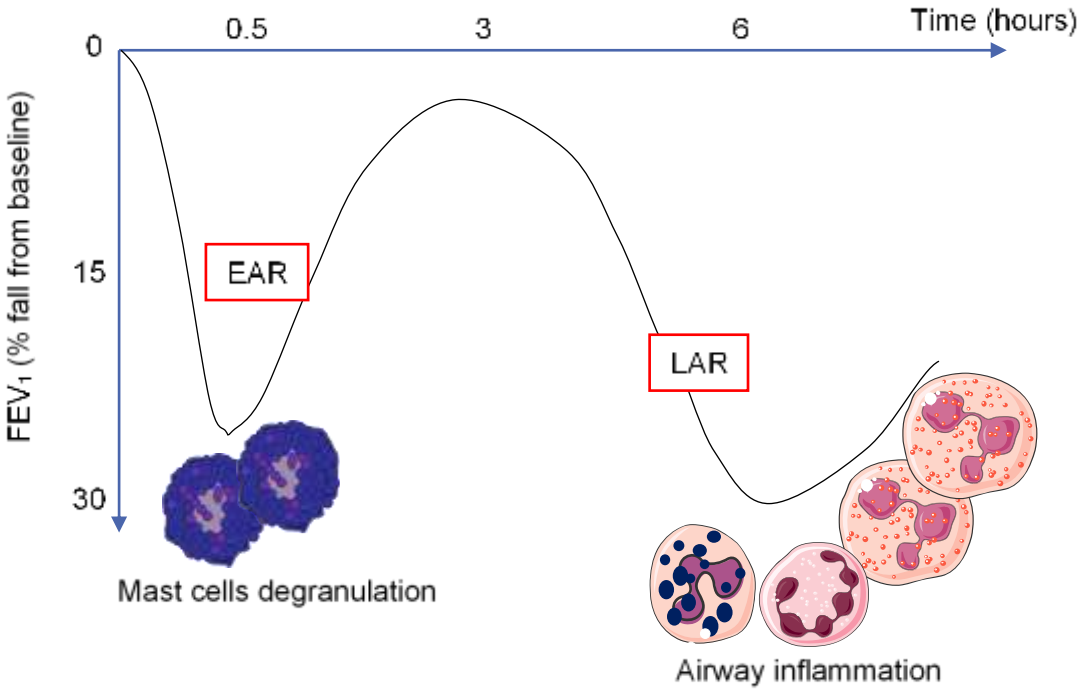


Figure 1-4 Kinetics of the EAR and LAR

Allergen challenge triggers an immediate drop in lung function named the early asthmatic response (EAR) provoked by mast cell degranulation, followed in half of the patients by another delayed and prolonged drop in lung function named the late asthmatic response (LAR) associated with an influx of inflammatory cells comprising eosinophils, basophils and neutrophils in the airways. Adapted from (Diamant et al. 2013; Lambrecht, Persson, and Hammad 2017).

1.4 The role of inflammatory cells in the late asthmatic response

The following sections present the current evidence on the involvement of inflammatory cells in the LAR. The therapies tested in allergen challenge studies in atopic asthmatics that are discussed in this thesis are summarised in (**Table 1**).

1.4.1 T cells

T cells are lymphoid-derived cells which mature in the thymus and continue their differentiation in lymph nodes. T cells are characterised by their expression of a T cell receptor (TCR) that recognises a specific antigen. After presentation of the antigen by antigen presenting cells (APCs) such as DCs, naïve T cells differentiate into different subsets depending on the cytokine environment. $CD4^+$ T cells recognise antigens presented on MHC II molecules and differentiate into T_H1, T_H2, T_H9, T_H17 helper cells or regulatory T cells (T_{reg}) that secrete distinct cytokines that orchestrate effector responses. T_{regs} have been shown to induce tolerance to a specific antigen, notably by secreting IL-10, IL-35 and transforming growth factor β (TGF-β) and suppressing T_H effector responses (Lambrecht, Hammad, and Fahy 2019; Shamji and Durham 2017). In contrast, $CD8^+$ T cells recognise antigens presented on MHC I molecules and differentiate into cytotoxic T cells that can secrete different cytokines and induce apoptosis in target cells (Pennock et al. 2013).

1.4.1.1 $CD4^+$ T_H2 cells as drivers of the LAR

$CD4^+$ T_H2 cells seem to predominate in atopic asthma and secrete the type-2 cytokines IL-4, IL-5 and IL-13. It is not clear whether allergen challenge is associated with an increase in $CD4^+$ T cell numbers in the airways, however, studies have consistently suggested T_H2 cells activation in the airways after allergen challenge (Bentley et al. 1993; D. Robinson et al. 1993).

One of the arguments in favour of T_H2 cells driving the LAR is the observation that immunosuppressive therapies inhibit the LAR in atopic asthmatics. As such, a single dose of inhaled corticosteroids significantly reduced the LAR in several studies (Cockcroft and Murdock 1987; Kidney et al. 1997; Parameswaran et al. 2000). Likewise, an inhibitor of T cell activation, cyclosporin A, inhibited the LAR, with a reduction in $CD4^+$ cell numbers in bronchial biopsies (Khan et al. 2000). A novel DNA enzyme that inactivates GATA3, the master transcription factor of T_H2 cytokines, also attenuated the LAR (Krug et al. 2015). In addition, the LAR can be modelled in atopic asthmatics by administering allergen epitopes that induce T cell activation in a HLA-dependent manner, further supporting a role for T cells in driving the LAR (Haselden, Kay, and Larché 1999; Ali et al. 2004).

While these therapies are not specific to T_H2 cells, animal models have provided the opportunity to specifically target $CD4^+$ T cells in the LAR. In two mouse OVA models, depleting $CD4^+$ cells with an anti-CD4 antibody abolished the LAR (Meyts et al. 2008; Nabe et al. 2011), a finding replicated in CD4-deficient mice (Baker et al., 2017). In addition, adoptive transfer of OVA-reactive $CD4^+$ T cells elicited a progressive and late-onset bronchospasm in rats and mice upon OVA challenge (A. Watanabe et al. 1995; Ohtomo et al. 2009). These data suggest that $CD4^+$ T_H2 cells are indeed important in driving the LAR.

1.4.1.2 Imbalance in $CD4^+$, $CD8^+$ T cells and T_{regs} subpopulations in the LAR

Concurrently, this predominance of $CD4^+$ T_H2 cells seems to be associated with a downregulation of $CD8^+$ and T_{regs} cell populations in the LAR. Indeed, a lower T_{reg} to $CD4^+$ cells ratio was found in the sputum of atopic asthmatics experiencing both an EAR and LAR (dual responders) compared to patients solely experiencing an EAR (single responders) (Kinoshita et al. 2014). Similarly, a lower $CD8^+$ to $CD4^+$ cell ratio was found in bronchoalveolar lavage (BAL) fluid (Gonzalez et al. 1987) as well as decreased numbers in IFN-γ^+ $CD8^+$ T cells in blood of dual responders compared to single responders (Yoshida et al. 2005). In fact, animal models have shown that $CD8^+\gamma\delta$ T cells producing IFN-γ played a protective role in the LAR by suppressing T_H2 cell function (Isogai et al. 2003; 2004; 2007; 2005; Baker et al. 2017). Overall, these data suggest that an imbalance in the ratio of inhibitory to pro-inflammatory T cells could drive the predominance of T_H2 cell population in the LAR and further support a role for $CD4^+$ T_H2 cells in driving the LAR.

1.4.1.3 Effector function of $CD4^+$ T_H2 cells in the LAR

Animal models have suggested that $CD4^+$ T_H2 cells orchestrate a cascade of events that leads to airway inflammation and airflow limitation by producing type-2 cytokines (Lambrecht and Hammad 2015). Indeed, adoptive transfer of OVA-primed $CD4^+$ T cells elicited an LAR-like bronchospasm in rats and this was abolished by pre-treating OVA-$CD4^+$ T cells with an anti-IL-4 antisense oligonucleotide (Molet et al. 1999). In another mouse OVA model, the LAR was attenuated by neutralising IL-13 (Taube et al. 2002). The development of monoclonal antibodies has allowed to test whether this was the case in human. So far, approaches targeting IL-13 cytokines have been disappointing on the LAR in mild atopic asthmatics. The monoclonal antibody IMA-026 specific for the IL-13 epitope binding IL-13Rα1 and IL-13Rα2 did not block the LAR. In contrast, IMA-638 (anrukinzumab) and lebrikizumab, two monoclonal antibodies specific for the IL-13 epitope binding IL-4Rα, seemed to reduce the LAR, although the effect of lebrikizumab was not statistically significant (Gauvreau et al. 2011; Scheerens et al. 2014). However, these mitigated results may be due to the redundancy of IL-4 and IL-13

actions. Indeed, an inhaled IL-4 mutein binding to IL-4Rα and acting as a competitive antagonist of IL-4/IL-13 named pitrakinra has demonstrated a 3.7 times reduction in the LAR (S. Wenzel et al. 2007). In addition, allergen challenge studies may not be appropriate for testing these targeted biologicals, as patients were not selected based on IL-4 or IL-13 engagement in the pathology (D. S. Robinson 2014). As such, retrospective analysis found a higher effect of lebrikizumab on the LAR in periostin-high patients, a biomarker depending on IL-13 biology (Scheerens et al. 2014).

Overall, the effect of IL-4Rα blockade on the LAR could indicate that IL-4 and IL-13 cytokines produced by T_H2 cells are involved in driving the LAR, but it remains unclear how these cytokines, and by extent T_H2 cells, lead to airflow limitation. In addition, these type-2 cytokines can also be produced by innate cells such as mast cells, basophils and ILC2s in varying abundance.

1.4.2 The epithelium – innate cell axis

As previously mentioned, mouse models using natural allergens such as HDM have revealed that innate immunity could also play an important role in triggering type-2 responses. In these models, allergen challenge stimulates epithelial cells and induces the rapid release of cytokines such as IL-33, TSLP and IL-25 that activate innate immune cells including basophils, mast cells and especially ILC2s to produce type-2 cytokines (Lambrecht, Hammad, and Fahy 2019). Concurrently, allergen challenge has been associated with an increase in the number of mucosal cells immunoreactive for IL-33, IL-25 and TSLP on bronchial biopsies (Al-Sajee et al. 2018; W. Wang et al. 2018) and in the levels of TSLP and IL-33 proteins in BAL fluid of mild atopic asthmatics (Winkler et al. 2019; Fux et al. 2014).

ILC2s reside in mucosal tissues and resemble T_H2 cells in their abundant expression of IL-5 and IL-13, but the difference is that ILC2 cells lack antigen specific receptors (van der Ploeg et al., 2020). Activated ILC2s expressing IL-5 and IL-13 have been found elevated in the BAL and sputum of atopic asthmatics at 24 h post-challenge, but no longer at 48 h in sputum (Winkler et al. 2019; Chen et al. 2017). This observation, conjoined with the rapid release of epithelial alarmins upon allergen challenge, with IL-33 found elevated within 10 min post-challenge (Fux et al. 2014), led to hypothesise that ILC2s could be responsible for the early recruitment of eosinophils to the lungs (Chen et al., 2017; Cosmi & Annunziato, 2017; van der Ploeg et al., 2020).

So far, ILC2s were shown to be involved in eosinophilia, goblet cell metaplasia and AHR in mouse models of allergic airway disease (Lambrecht, Hammad, and Fahy 2019), yet their role has not been explored in the LAR. Nonetheless, a recent study demonstrated that an anti-

TSLP monoclonal antibody, tezepelumab, attenuated both EAR and LAR in atopic asthmatics (Gauvreau et al. 2014). In addition, human ILC2s are characterised by their expression of the PGD_2 receptor CRTH2 and PGD_2 is needed for their activation (Maric et al., 2019; van der Ploeg et al., 2020). Two CRTH2 antagonists were found to reduce the LAR without affecting the EAR in atopic asthmatics (Diamant et al. 2014; Singh et al. 2013). Therefore, these data could indicate a role for ILC2 cells, and more globally for the epithelium-innate cell axis, in driving the LAR.

1.4.3 Eosinophils

Whether produced by T_H2 cells or other innate cells, type-2 cytokines are characteristically associated with airway eosinophilia (Lambrecht and Hammad 2015). Eosinophils are granulocytes that develop in the bone marrow from a myeloid progenitor and circulate in the blood as differentiated cells with a short life span. These cells are recruited in the airways by eotaxins and IL-5 promotes their survival in inflamed tissues (Lambrecht, Persson, and Hammad 2017). It has been a common assumption that recruited eosinophils are responsible for causing airflow limitation in the LAR, based on the observation that corticosteroids potently inhibit the LAR and associated airway eosinophilia (Gauvreau et al. 1996; Inman et al. 2001; Leigh et al. 2002; Becky Kelly, Busse, and Jarjour 2000; Parameswaran et al. 2000; Diamant et al. 2013). The effector function of eosinophils is attributed to their secretion of mediators that act on structural cells (Lambrecht, Persson, and Hammad 2017). Because eosinophils secrete CysLTs (Weller et al. 1983) and LTRAs were reported to reduce the LAR in atopic asthmatics (Roquet et al. 1997; Davis et al. 2009; Leigh et al. 2002), it has been hypothesised that recruited eosinophils could be responsible for a second wave of CysLTs after the EAR causing the LAR (Diamant et al. 2013).

However, the assumption of a causal link between airway eosinophilia and airflow obstruction has been disputed. Patients with asthma were reported to exhibit the same degree of eosinophil infiltration than patients with Eosinophilic Bronchitis (EB), while EB is not associated with variable airflow limitation (Brightling, Bradding, et al. 2002; Brightling et al. 2003). In addition, animal models have shown that it is possible to induce prolonged airflow limitation to allergen exposure in the absence of eosinophil influx (Mizutani et al. 2012; A. Watanabe et al. 1995) or an influx peaking later than airflow limitation (Meyts et al. 2008; S. L. Underwood et al. 2002). In human, it is less clear when eosinophilic inflammation peaks, but two time-course studies following patients across different timepoints have reported a peak between 24 and 42 h post-challenge in BAL fluid or sputum (Lommatzsch et al. 2006; Gauvreau, Watson, and O'Byrne 1999).

IL-5 is a crucial driver of airway eosinophilia and the development of anti-IL-5 monoclonal antibodies has enabled to test the contribution of eosinophils in the LAR. So far, only mepolizumab (anti-IL-5) has been tested in an allergen challenge study (Leckie et al. 2000), with an ongoing trial for benralizumab (anti-IL-5Rα) (NCT02821416). However, in spite of preventing blood eosinophilia after allergen challenge, mepolizumab did not prevent the LAR in atopic asthmatics (Leckie et al. 2000). As for anti-IL-4/IL-13 therapies, this may be due to the non-selective design of allergen challenge studies. It has also been proposed that mepolizumab did not block the LAR because of an insufficient reduction in tissue eosinophils, a hypothesis which could be evaluated by the ongoing trial of benralizumab which has a reported quicker and more profound reduction in tissue eosinophils (Lambrecht, Hammad, and Fahy 2019; Kay and Menzies-Gow 2003). It has also been suggested that a combination therapy blocking IL-3, IL-5, GM-CSF and CCR3 receptors all involved in eosinophilopoiesis might be more efficient (Kay and Menzies-Gow 2003), but this hypothesis remains unanswered as both a CCR3 antagonist or antisense therapy blocking IL-3, IL-5, GM-CSF and CCR3 receptors did not reduce the LAR but also did not efficiently reduce airway eosinophil numbers (Gauvreau et al. 2008; 2018). Nonetheless, this failure of mepolizumab to block the LAR has led scientists to question the assumption of a causal link between eosinophilic inflammation and variable airflow limitation (Cockcroft 2014).

1.4.4 Role of IgE-dependent mechanisms

Atopic asthma is associated with the production of allergen-specific IgE, a hallmark of T_H2 inflammation. While it is admitted that IgE is an essential trigger of the EAR, the dependence of the LAR on IgE-dependent mechanisms has been originally disputed. Indeed, the correlation between total or specific IgE levels and the LAR has been controverted (Stokes Peebles et al. 2001; Durham et al. 1984) and only half of the patients experiencing an EAR develop an LAR (Diamant et al. 2013). In addition, administration of allergen-derived T cell epitopes has elicited LAR-like responses in atopic asthmatics independently from an IgE mechanism or developing an EAR (Haselden, Kay, and Larché 1999; Ali et al. 2004). However, neutralising free IgE molecules with the monoclonal antibody omalizumab effectively reduced both EAR and LAR by 85% and 65% in atopic asthmatics, suggesting that IgE-dependent mechanisms are indeed involved in the LAR (Fahy et al. 1997).

1.4.5 Mast cells and basophils

IgE binds to high-affinity FcεRI receptors constitutively expressed on mast cells and basophils and allergen crosslinking of IgE/FcεRI complexes leads to mast cells and basophils activation and the release of bronchoconstrictor mediators such as CysLTs. Treatment with omalizumab has been associated with a fast downregulation of FcεRI receptors on mast cells and basophils, which could be a possible mechanism by which omalizumab reduces airflow limitation (Holgate et al. 2005). As both mast cells and basophils release similar mediators and so far no therapy has specifically targeted either cell type, it remains difficult to determine their relative contribution in the LAR. Therefore, the role of both cell types will be discussed in this section.

Mast cells are long-lived granulated metachromatic cells residing in human tissues. These cells develop from a myeloid progenitor in the bone marrow and terminally differentiate in tissues under influences from the local environment. The differentiated cells contain numerous granules storing preformed mediators, such as histamine or proteases including the abundant mast cell tryptase used as a mast cell marker, and secrete a wide range of lipid mediators such as CysLTs (LTC_4, LTD_4, LTE_4) and prostaglandins (PGD_2) that are potent inducers of bronchospasm, mucus secretion and vascular leakage (Bradding and Arthur 2016). The allergen crosslinking IgE-FcεRI complexes on tissue-resident mast cells is unanimously believed to provoke the EAR. Indeed, allergen challenge is followed within minutes by the rapid elevation of tryptase and PGD_2 in the airways, which are mediators mostly produced by mast cells. However, the involvement of mast cells in the LAR has been disputed on the observation that PGD_2 and tryptase were found decreased or undetected in the BAL of

asthmatic patients at later timepoints from 12 to 48 h post-allergen challenge (O'Sullivan et al. 1998; M. C. Liu et al. 1991; Sedgwick et al. 1991; S. E. Wenzel, Fowler, and Schwartz 1988).

Basophils are granulocytes derived from a myeloid progenitor in the bone marrow and resemble mast cells with their FcεRI expression, metachromatic staining and secretion of mediators such as CysLTs and histamine, although mast cells seem to display a wider range of mediators. Unlike mast cells, basophils circulate in the blood as terminally differentiated cells with a short life span and can be recruited in inflamed tissues (Lambrecht, Persson, and Hammad 2017). An influx of basophils has been observed in the airways of atopic asthmatics within the timeframe of the LAR, with a peak at 7 h post-challenge and a higher increase in dual responders compared to single responders (Gauvreau et al. 2000). A single dose of corticosteroids has been shown to reduce this basophil influx (M. C. Liu et al. 2001).

In addition to reducing the EAR, antagonists of CysLT receptors (LTRAs) inhibited the LAR in mild atopic asthmatics in several allergen challenge studies, with reductions in maximum FEV_1 fall between 30 and 63% compared to placebo (Leigh et al. 2002; Davis et al. 2009; Roquet et al. 1997; Diamant et al. 1999; Hamilton et al. 1998). Antagonists of histamine H1 receptors also reduced the LAR but with a more modest effect (Roquet et al. 1997; Davis et al. 2009) and the combination of LTRAs and antihistamines was associated with a 74 to 88% reduction (Roquet et al. 1997; Davis et al. 2009). Therefore, it has been hypothesised that mast cells degranulation provokes the EAR, following which the recruitment of basophils and their activation in the airways by an IgE-dependent mechanism leads to a second wave of mediators such as CysLTs causing the LAR (Gauvreau, El-Gammal, and O'Byrne 2015). However, a study reported that basophil infiltration represented less than 10% of infiltrating eosinophils in the bronchial mucosa of asthmatic subjects during the late phase response, while it represented approximately 40% in cutaneous late phase responses (Macfarlane et al. 2000). Therefore, the relative importance of basophilic inflammation remains to be established in the LAR.

In addition, the decrease of the mast cell markers PGD_2 and tryptase may not necessarily imply that mast cells are not involved in the LAR, as mast cells can release different mediators at different timepoints (Bradding and Arthur 2016). CRTH2 antagonists (PGD_2 receptor antagonists) and a tryptase inhibitor were found to reduce the LAR without affecting the EAR in atopic asthmatics (Diamant et al. 2014; Singh et al. 2013; Krishna et al. 2001). Therefore, mast cell activation during the EAR could induce the release of mediators that initiate the LAR (Bradding and Arthur 2016).

In fact, mast cells have been considered as key cells in the development of airflow limitation in asthma. Indeed, mast cell infiltration in airway smooth muscle (ASM) bundles is a

characteristic feature of asthma (Brightling, Bradding, et al. 2002). As previously described, patients with Eosinophilic Bronchitis (EB) and patients with asthma present similar eosinophil infiltration and elevation of T_H2 cytokines, but EB is not associated with variable airflow obstruction. However, ASM bundles are characteristically infiltrated by mast cells in asthma but not in EB, suggesting that mast cells situated in ASM bundles are key cells in airway obstruction processes in asthma (Brightling, Bradding, et al. 2002; Brightling, Symon, et al. 2002; Brightling et al. 2003).

Overall, mast cells and basophils could be involved in the LAR by releasing mediators such as CysLTs and histamine that contract the airway smooth muscle. However, it is important to note that the magnitude of LAR reduction exerted by LTRAs seems variable between studies, with a higher reduction in studies with patients exhibiting milder LAR responses in the placebo arm (Leigh et al. 2002; Davis et al. 2009; Roquet et al. 1997; Diamant et al. 1999; Hamilton et al. 1998). This variability seems to be found in the clinic, with heterogeneous responses to LTRAs as add-on controller therapy between asthmatic patients (Szefler et al. 2005). Furthermore, antihistamines are poorly efficacious in the clinic (O'Byrne 2009). Therefore, it is important to investigate alternative mechanisms of airflow limitation than CysLTs and histamine-mediated bronchospasm.

Interestingly, mast cells have been shown to interact closely with airway nerves. In animal models of allergy, mast cells degranulation upon allergen challenge increased the excitability of airway sensory and parasympathetic nerves, an effect lasting for hours after allergen challenge (Undem and Taylor-Clark 2014; Bradding and Arthur 2016), further corroborating the notion that activation of mast cells during the EAR could initiate later events leading to the LAR. In fact, interactions between mast cells and nerves have been hypothesised to play an important role in triggering airflow limitation in asthma (Undem and Taylor-Clark 2014). Therefore, investigating the role of airway nerves could lead to discover new mechanisms triggering the LAR, and by extent airflow limitation in atopic asthma.

	Compound	EAR	LAR	Eosinophils	References
LABA	Formoterol Salmeterol	↘↘	↘↘	N.S. or ↘	(Duong et al. 2007; B. Pedersen et al. 1993; Pizzichini et al. 1996; Dente et al. 1999; I. K. Taylor et al. 1992)
ICS	Budesonide Mometasone Fluticasone Beclometha-sone	N.S. or ↘ with regular treatment	↘↘	↘↘	(Gauvreau et al. 1996; Kidney et al. 1997; Inman et al. 2001; Leigh et al. 2002; Burge et al. 1982; Cockcroft and Murdock 1987)
Anti-H1R	Loratadine Desloratadine	↘	↘	↘ at 7h	(Roquet et al. 1997; Davis et al. 2009)
LTRA	Zarfilukast Montelukast Pranlukast	↘↘	↘	N.S. or ↘	(Hamilton et al. 1998; Leigh et al. 2002; I. K. Taylor et al. 1992; Davis et al. 2009; Diamant et al. 1999; Roquet et al. 1997)
Anti-IgE	Omalizumab	↘	↘	N/A	(Fahy et al. 1997; Trischler et al. 2017)
Anti-IL-5	Mepolizumab	N.S.	N.S.	↘↘	(Leckie et al. 2000)
Anti-IL-13	Lebrikizumab IMA-638 IMA-026	N.S. or ↘	N.S. or ↘	N/A or N.S.	(Scheerens et al. 2014; Gauvreau et al. 2011)
Anti-IL-4Rα	Pitrakinra	N.S.	↘	N.S.	(S. Wenzel et al. 2007)
Anti-TSLP	Tezepelumab	↘	↘	↘	(Gauvreau et al. 2014)
Anti-CRTH2	Setipiprant Timapiprant	N.S.	↘	N.S. or ↘	(Diamant et al. 2014; Singh et al. 2013)
GATA-3 DNAzyme	SB010	↘	↘	N.S.	(Krug et al. 2015)
CCR3, IL-3, IL-5, GM-CSF inhibition	TPI ASM8 AXP1275	N.S. or ↘	N.S.	N.S.	(Gauvreau et al. 2008; 2018)
Tryptase inhibitor	APC366	N.S.	↘	N/A	(Krishna et al. 2001)

Table 1 Allergen challenge studies in mild atopic asthmatics discussed in this thesis

Based upon (Gauvreau, El-Gammal, and O'Byrne 2015; D. S. Robinson 2014; Hilvering and Pavord 2016). ↘: decrease in the parameter. Eosinophils: measured in blood or airway compartment. N/A: not measured. N.S.: effect not statistically significant.

1.5 The role of airway nerves in the late asthmatic response

Given previously discussed studies, it is still unclear which actions of inflammatory cells are responsible for causing airflow limitation in the LAR and more generally in atopic asthma. Before the discovery of corticosteroids and advances in immunology, asthma was considered as an alteration of the nervous system (Mazzone and Undem 2016). In fact, airway tone is predominantly governed by a parasympathetic reflex (Canning and Fischer 2001; Canning 2006). Airway sensory nerves can be activated by a multitude of stimuli including inflammatory mediators and this can trigger parasympathetic cholinergic nerves to release acetylcholine (ACh) that stimulates airway smooth muscle cells to contract, epithelial glands to secrete mucus and vessels to dilate. Therefore, it has been hypothesised that airway nerves could be the missing link between inflammation and allergy symptoms (Undem and Taylor-Clark 2014). This section presents basic principles of airway innervation and the current evidence on the involvement of nerves in asthma and the LAR.

1.5.1 Airway innervation

1.5.1.1 Physiology of airway reflexes

Breathing is an automatic function controlled by a network of neurons in the brainstem. On top of this, the respiratory system is constantly adjusting to chemical and mechanical pressures thanks to a dense sensory innervation. This functions as a reflex arc: afferent (sensory) nerves sense changes in local environment and communicate the information to integration centres in the central nervous system (CNS), which produce an output signal in efferent nerves that interact with effector cells such as skeletal, smooth muscle cells and glands**,** thereby regulating breathing and triggering protective reflexes such as cough and bronchospasm (Mazzone and Undem 2016) (**Figure 1-5**).

The main nerve innervating the airways is the vagus nerve, which contains both afferent and efferent fibres. Two vagal nerve trunks project to the respiratory tract on each side of the body via distinct branches: the superior laryngeal nerve (SLN) innervating the larynx, the recurrent laryngeal nerve (RLN) the trachea, and pulmonary branches further bronchi and lung parenchyma (Mazzone and Undem 2016) (**Figure 1-5**). Past and around the airways, the vagus nerve innervates other organs including the heart and digestive tract. Early electronic microscopy studies found a higher density of nerves in the large airways (Jeffery and Reid 1973; A. Laitinen 1985), however, nerves are found as far as terminal bronchioles (Larson et al. 2003).

Airway sensory neurons (airway vagal afferent nerves) sense changes in the airway environment thanks to receptors present on their airway terminals. These neurons are pseudo-unipolar: their axons transmit action potentials from the airways to their central terminals in the brainstem, mostly located in the *Nucleus Tractus Solitarii* (NTS). Their cell bodies containing their nuclei are localised in the nodose or jugular ganglia at the base of the skull (Mazzone and Undem 2016) (**Figure 1-5**).

While afferent nerves constantly send information to the CNS about the state of the airways, efferent nerves constantly send commands back to the respiratory tract. A dense innervation of efferent cholinergic parasympathetic nerves controls the tone of conducting airways, with its highest density in the large airways (Wine 2007). Parasympathetic preganglionic nerves originate from the *Nucleus Ambiguus* (NA) and *Dorsal Motor Nucleus* (DMN) in the brainstem and travel through the vagus nerve to parasympathetic ganglia in the vicinity of the airways. In these ganglia, preganglionic nerves synapse with postganglionic parasympathetic fibres that innervate airway smooth muscle cells, submucosal glands and vessels. Once stimulated, cholinergic postganglionic neurons release acetylcholine (ACh) which activates muscarinic receptors on effector cells and triggers bronchoconstriction and mucus secretion (Undem and Potenzieri 2012) (**Figure 1-5**).

The sympathetic system is often described as the antagonist of the parasympathetic system. Surprisingly, airways of most species are scarcely innervated by sympathetic nerves, with few exceptions such as guinea pigs (Richardson and Beland 1976; Undem and Potenzieri 2012). Airway smooth muscle relaxation is rather mediated by the non-adrenergic non-cholinergic (NANC) system, with studies showing that NO is the predominant relaxant of human airways (Belvisi et al. 1992; Undem and Potenzieri 2012). However, while sympathetic nerves are scarcely found, adrenergic receptors are highly expressed in the airways, with a high density of β2 adrenergic receptors (Carstairs, Nimmo, and Barnes 1985). These GPCRs are coupled to G_s and their activation induces airway smooth muscle cells relaxation, a finding highly exploited in asthma with the potent bronchodilators β2-agonists LABAs and SABAs.

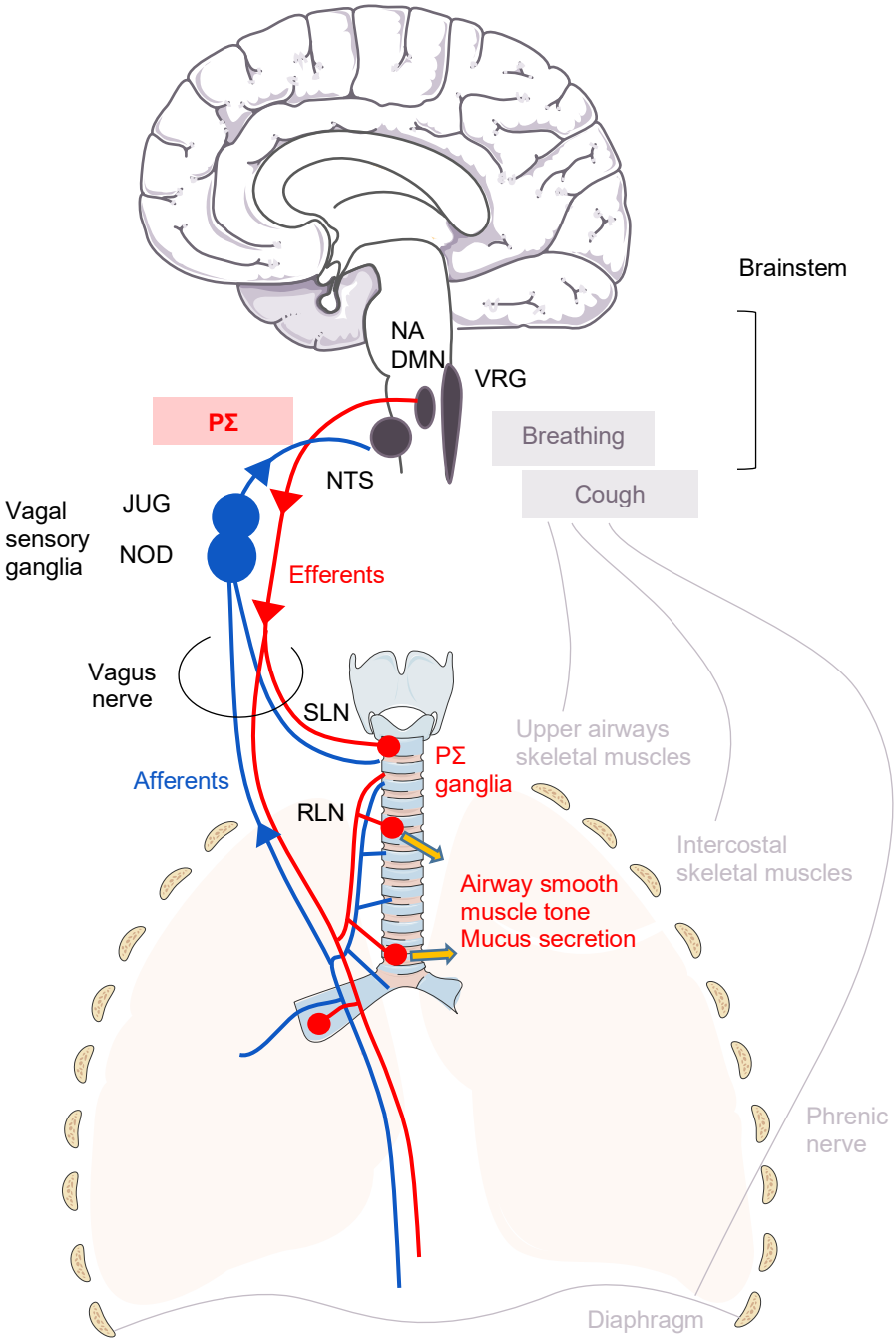


Figure 1-5 Airway innervation and reflexes

Airway afferent (sensory) fibres are stimulated by chemical or mechanical stimuli in the airways (blue lines). The information is conveyed to integration centres in the CNS. The output can modulate breathing and provoke protective reflexes such as cough and cholinergic reflexes. In the case of a cholinergic reflex, efferent parasympathetic cholinergic fibres (red lines) are activated and release ACh in the airways, provoking reflex bronchospasm and mucus secretion. Based on (Mazzone and Undem 2016; Grace et al. 2013). DMN: Dorsal Motor Nucleus; JUG: Jugular ganglia; NA: Nucleus Ambiguus; NOD: Nodose ganglia; NTS: Nucleus of the Solitary Tract; PΣ: Parasympathetic; RLN: Recurrent Laryngeal Nerve; SLN: Superior Laryngeal Nerve; VRG: Ventral Respiratory Group.

1.5.1.1 Electrophysiology of nerve activation

Neurons convey information in the form of action potentials propagating through their axons. Chemical or mechanical stimuli can open ion channels on the neuronal membrane and lead to depolarisation. If the depolarisation reaches the threshold of activation of voltage-gated sodium channels (Na_v), this provokes an action potential: Na_v channels open, leading to an influx of Na^+ and a rapid depolarisation, following which Na_v inactivate and voltage-gated potassium channels (K_v) activate, leading to an efflux of K^+ and repolarisation of the membrane. This local action potential depolarises the adjacent axon membrane and this leads to the propagation of action potentials down the axon in a single direction (Barnett and Larkman 2007).

A nerve contains different neurons which can be differentially activated. The sum of action potentials recorded in a nerve is called “compound action potential”. Action potentials propagate along axons at different speeds depending on the axon proprieties: axons with a large diameter and electrically insulated by a gain of myelin possess fast conduction velocities, while unmyelinated axons with a small diameter exhibit slower conduction velocities. Electrically stimulating a nerve can activate axons present within this nerve and produce a compound action potential usually in three waves: the Aβ, Aδ and C-wave, from fastest to slowest as shown in (**Figure 1-6**). The Aβ and Aδ wave are comprised of action potentials propagated in fast conducting axons while the C-wave is comprised of potentials propagated in slow conducting axons. By extent, neurons have been classified according to their conduction velocity in the Aβ, Aδ and C-range (Mazzone and Undem 2016).

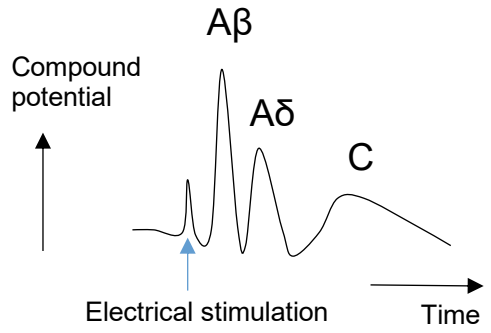


Figure 1-6 Assessment of airway nerves conduction velocity

To assess conduction velocity, the whole nerve is electrically stimulated and compound action potential firing recorded over time with a set of electrodes located at distance of the stimulation. Electrical stimulation produces a compound action potential usually in three waves, from fastest to slowest: Aβ, Aδ and C-wave, constituted by individual action potentials propagated in fast (Aβ, Aδ-range) and slow (C-range) conducting axons. Adapted from (Mazzone and Undem 2016).

1.5.2 Vagal afferent nerves

Different types of afferent nerve fibres have been classified based on their conduction velocity, response to stimuli and location within the airways (Mazzone and Undem 2016). Extensive studies have also classified afferent fibres according to their cell body location in the nodose or jugular ganglia (Ricco et al. 1996; Undem et al. 2004; Kajekar et al. 1999; Chuaychoo et al. 2005; 2006; Kwong et al. 2008). Due to the technical difficulty of studying nerves in human, this classification is mostly based on anaesthetised or *in vitro* preparations conducted in guinea pigs, as the morphology of guinea pig airway innervation presents similarities with that of humans (West et al. 2015). So far, four main subtypes of vagal afferent fibres have been described and this classification is summarised in (**Table 2**).

1.5.2.1 Slowly adapting receptors (SARs)

Slowly adapting receptors (SARs) are myelinated fibres derived mostly from the nodose ganglia that conduct in the fast Aδ-range (Schelegle and Green 2001; Canning et al. 2004). SARs play an important role in the regulation of breathing and were originally described as stretch receptors responsible for preventing lung over-inflation as part of the “Hering-Breuer reflex” (H. M. Coleridge, Coleridge, and Schultz 1989). SARs are mostly found in the lungs associated with the airway smooth muscle layer (Bartlett et al. 1976). These fibres possess a low mechanical threshold and are characteristically activated by lung distension: SARs fire each time inspiration occurs, as long as lung inflation is maintained (Bartlett, Sant’ambrogio, and Wise 1976). At peak inflation, SARs discharge switches off inspiratory centres in the NTS and inhibits cholinergic drive, promoting expiration and bronchodilation (Schelegle and Green 2001; Mazzone and Undem 2016; Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002a).

1.5.2.2 Rapidly adapting receptors (RARs)

Rapidly adapting receptors (RARs) are myelinated fibres derived mostly from the nodose ganglia that conduct in the fast Aδ-range (Ricco et al. 1996; Canning et al. 2004; Adcock et al. 2014). RARs are also stretch receptors with a low mechanical threshold and are thus very active during normal ventilation, with the difference that RARs quickly adapt to lung inflation and their discharge rapidly stops at the beginning of inspiration (Bergren 1997; Bergren and Sampson 1982; Sant’Ambrogio et al. 1978). RARs are found within the mucosa, with a higher density in large airways (Canning et al. 2004; Sant’Ambrogio et al. 1978; Mortola, Sant’ambrogio, and Clement 1975).

RARs seem to respond to any mechanical pressure applied to the respiratory tract, such as lung inflation/deflation, bronchoconstriction, but also oedema and mucus secretion (Bergren 1997; Bergren and Sampson 1982; Sant'Ambrogio et al. 1978; Canning et al. 2004; Widdicombe 2003; Bonham et al. 1996; Kappagoda and Ravi 1989). Accordingly, RARs were shown to be strongly activated upon challenge with potent bronchoconstrictors such as LTD_4 or secondary to reflex bronchospasm caused by inflammatory mediators activating airway nerves (Bergren 1997; Canning et al. 2004). Recent findings suggested that RARs could in fact be activated by the release of adenosine triphosphate (ATP) that is generated by mechanical stretch, with ATP activating P2X2/3 purinergic receptors on RAR fibres (Canning et al. 2004; Weigand, Ford, and Undem 2012).

RARs have generally not been shown to be directly activated by chemicals, however studies have reported subsets of RARs responsive to chemicals such as capsaicin or acids (Widdicombe 2003; Ho et al. 2001; Kollarik and Undem 2002; Adcock et al. 2014). In contrast to SARs, RAR axons terminating within the NTS seem to promote excitatory responses, including tachypnea, cough and potentially cholinergic bronchospasm and mucus secretion (Widdicombe 2003; Chou et al. 2008; Tatar, Sant'Ambrogio, and Sant'Ambrogio 1994; Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002a; J. Yu et al. 1989).

1.5.2.3 C-fibres

C-fibres are unmyelinated afferent fibres that conduct in the slow C-range, with conduction velocities inferior to 1 m/s (Fox et al. 1993; Ricco et al. 1996). C-fibres appear to form a varicose network in the airway epithelial layer (Larson et al. 2003; West et al. 2015) and are described as chemosensitive fibres. Indeed, C-fibres appear only weakly activated by stretch or lung inflation and present sparse spontaneous activity at eupneic breathing but are characteristically activated by irritants such as capsaicin, cigarette smoke components and autacoids such as bradykinin (J. C. Coleridge and Coleridge 1984; Ho et al. 2001; Fox et al. 1993; Ricco et al. 1996; Bergren 1997; Undem et al. 2004; Kollarik and Undem 2002; Kajekar et al. 1999). The observation that these fibres were normally quiescent but activated by noxious substances led to hypothesise that C-fibres are nociceptors conveying the information that lungs are exhibiting damage (Widdicombe 1982).

C-fibres seem to be directly activated by chemical compounds, as the cognate receptors of compounds were found directly expressed on C-fibre neurons (Ho et al. 2001; Bergren 1997; Undem et al. 2004; Chuaychoo et al. 2006; 2005), such as TRPV1 for capsaicin (Kwong et al. 2008; Nassenstein et al. 2010). In fact, C-fibres are commonly identified by their capsaicin response and/or their TRPV1 expression, but capsaicin-insensitive C-fibres also exist (Nasra and Belvisi 2009; Mazzone and Undem 2016; Kollarik et al. 2003; Adcock et al. 2014).

Interestingly, findings in guinea pigs and rodents have suggested that the phenotype of C-fibres mostly depends on whether their cell body is localised in the nodose or jugular ganglia (Undem et al. 2004; Kwong et al. 2008; Nassenstein et al. 2010; Kollarik, Ru, and Undem 2019):

1.5.2.4 Jugular C-fibres

Jugular C-fibres appear to be the most prominent C-fibres at the tracheal level (Ricco et al. 1996; Hunter and Undem 1999; Undem et al. 2004) and express neuropeptides such as the tachykinins substance P and neurokinin A (NKA) (Hunter and Undem 1999; Ricco et al. 1996). These neuropeptides can be released at the site of C-fibre stimulation or at distal branches independently from a central reflex ("local axon reflex") and elicit vascular leakage, smooth muscle contraction and inflammation, a process named neurogenic inflammation (Barnes, Baraniuk, and Belvisi 1991). However, these neuropeptides are found highly expressed in guinea pigs but less so in human (Lamb and Sparrow 2002). Jugular C-fibres activation within the upper airways has been shown to induce apnoea and slow breathing and to provoke bronchospasm and mucus secretion, either as a cholinergic or tachykinin-mediated reflex, as well as cough (Chou et al. 2008; Bergren 1988; Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002a; Fuller, Dixon, and Barnes 1985; L. Y. Lee and Pisarri 2001; Forsberg and Karlsson 1986; Laude, Higgins, and Morice 1993).

1.5.2.5 Nodose C-fibres

Nodose C-fibres are mostly found in intrapulmonary locations (Undem et al. 2004). In contrast to jugular C-fibres, less nodose C-fibres are found to express neuropeptides (Undem et al. 2004). Nodose C-fibres are chemosensitive to stimuli activating jugular C-fibres such as capsaicin and bradykinin, but also respond to different stimuli such as 5-HT (Kajekar et al. 1999; Undem et al. 2004; Kollarik and Undem 2002; Chuaychoo et al. 2005). While jugular C-fibres are poorly mechanosensitive, nodose C-fibres were shown to be activated by bronchoconstriction, an effect indirectly mediated by the generation of ATP activating P2X2/3 receptors (Weigand, Ford, and Undem 2012). Nodose C-fibres have been shown to induce tachypnea in guinea pigs and may inhibit cough (Chou et al. 2008; Chou, Mori, and Canning 2018).

	SARs	RARs	Jugular C-fibres	Nodose C-fibres
Fibre type	Aδ myelinated	Aδ myelinated	C unmyelinated	
Conduction velocity	~ 18 m/s	~ 15 m/s	< 1 m/s	
Ganglia	nodose	nodose	jugular	nodose
Location intra/extra pulmonary	Intra>extra Small airways	Intra>extra Large airways	Extra>intra	Intra>extra
Mechanical sensitivity	+++ Touch Stretch Bronchospasm	+++ Touch Stretch Bronchospasm	-	++
Chemical sensitivity	-	- Acid	+++ Acid Caps, BK PGE_2	+++ Acid Caps, BK PGE_2, 5-HT
Neuropeptides	-	-	+++	+
Physiological response	Inhibits inspiration ↘ PΣ: Bronchodilation	Tachypnea ↗ PΣ: Bronchospasm, Mucus secretion Cough	Apnoea ↗ PΣ: Bronchospasm, Mucus secretion Cough	Tachypnea ↗ PΣ: Bronchospasm, Mucus secretion Inhibits Cough

Table 2 Classification of vagal airway afferent fibres

References from (Fox et al. 1993; Ricco et al. 1996; Undem et al. 2004; Canning et al. 2004; Schelegle and Green 2001; Widdicombe 2003; Kollarik and Undem 2002; Kajekar et al. 1999; Chou et al. 2008; Nasra and Belvisi 2009; Chou, Mori, and Canning 2018; Mazzone and Undem 2016; Chuaychoo et al. 2005; Kwong et al. 2008). BK: bradykinin; Caps: capsaicin; ↗ ↘ PΣ: activation or inhibition of parasympathetic cholinergic drive; SARs: Slowly Adapting Receptors; RARs: Rapidly Adapting Receptors.

1.5.3 Vagal efferent cholinergic nerves

The airways of a healthy subject are constantly contracting with a baseline cholinergic tone. According to animal studies, this originates from a central reflex fed by constant inputs from mechanosensitive intrapulmonary vagal afferents, which are potentially RAR fibres (Canning 2006; Widdicombe 1966; Mitchell, Herbert, and Baker 1985; Kesler and Canning 1999; Jammes and Mei 1979; Weigand, Ford, and Undem 2012). An early study suggested that it is also the case in human, as bronchi from healthy subjects contract upon breath holding but this does not happen in double lung transplant subjects who have their airway innervation cut below the mainstem bronchi (Molfino et al. 1993).

Cholinergic postganglionic neurons release ACh that activates M1, M2 and M3 muscarinic receptors. M3 are G protein coupled receptors (GPCRs) coupled with G_q that are expressed on airway smooth muscle cells and submucosal glands and their activation provokes airway smooth muscle contraction and mucus secretion (Watson, Magnussen, and Rabe 1995; D'Agostino et al. 2008; Rogers 2001). M1 are coupled with G_q and are expressed by epithelial cells and their activation regulates electrolytes flux and facilitates parasympathetic transmission (Gosens and Gross 2018). In contrast, M2 are coupled with G_i and are more expressed than M3 receptors in the airways. M2 are notably expressed on postganglionic terminals and act as autoinhibitory receptors: after being released, ACh activates M2 receptors present on cholinergic nerve terminals and this diminishes ACh release, potentially limiting airway smooth muscle contraction and mucus secretion (Patel et al., 1995; ten Berge, Zaagsma, & Roffel, 1996).

Therefore, airways are challenged at each inspiration by ACh released from cholinergic postganglionic nerves and activating muscarinic receptors, thus maintaining a baseline tone. Stimulation of Aδ or C-fibre airway afferents by mechanical stimuli or inflammatory mediators can enhance this cholinergic tone and result in reflex bronchospasm and mucus secretion, as described in **1.5.2**. Several lines of evidence have suggested that this cholinergic tone could be enhanced in asthmatic patients and be responsible for the excessive bronchospasm seen in asthma and the LAR.

1.5.4 Airway nerves in the late asthmatic response

1.5.4.1 Evidence of a cholinergic reflex in the LAR

Early studies have suggested that vagal cholinergic tone is enhanced in asthmatic patients. As previously described, the bronchi of healthy subjects contract during breath holding and this is caused by a physiological vagal cholinergic reflex. In a small study, the bronchi of asthmatic patients contracted more than healthy controls during voluntary apnoea, and this effect was blocked by the antimuscarinic ipratropium, suggesting the existence of an enhanced vagal cholinergic tone in asthmatic patients (Molfino et al. 1993). The measurement of cardiac parameters also suggested an overall increase in vagal cholinergic tone in atopic asthmatics (Hashimoto, Maeda, and Yokoyama 1996).

Anticholinergics have not been used in asthma for decades, on the basis that short-acting muscarinic antagonists (SAMAs) such as ipratropium were shown to be less effective than β2-agonists in improving lung function in asthma (Westby, Benson, and Gibson 2004). In allergen challenge studies, atropine and ipratropium were associated with only a mild effect (D. Y. Yu, Galant, and Gold 1972; Cockcroft, Ruffin, and Hargreave 1978) or an absence of effect on the EAR (Rosenthal et al. 1977; Ruffin, Cockcroft, and Hargreave 1978). Similarly, ipratropium did not improve the LAR in atopic asthmatics (Cockcroft, Ruffin, and Hargreave 1978). Nonetheless, it has been hypothesised that SAMAs were not effective on reducing allergen-induced bronchoconstriction due to their short half-life and inhibition of M2 auto-inhibitory receptors (Cazzola, Centanni, and Donner 1998). Indeed, SAMAs are non-selective M1/M2/M3 antagonists and the inhibition of M2 receptors on postganglionic cholinergic nerves has been associated with an enhanced ACh release and airway smooth muscle contraction. As such, ipratropium enhanced ACh release *in vitro* in human trachea (Patel et al., 1995; ten Berge, Zaagsma, & Roffel, 1996). This hypothesis could however be challenged by the observation that M2 receptors seem to be downregulated in asthmatic airways (Minette et al. 1989).

In contrast to ipratropium, tiotropium is a long-acting muscarinic antagonist (LAMA) and a potent M1/M2/M3 muscarinic antagonist which dissociates more quickly from M2 than from M1 and M3 receptors (Casarosa et al. 2009). Interestingly, tiotropium was recently found effective on improving symptoms and baseline lung function in asthmatics patients and is now approved as add-on controller therapy (Buhl and Hamelmann 2019; Paggiaro et al. 2016; Kerstjens et al. 2015; 2012). On-going trials for the alternate LAMA glycopyrrolate show similar findings (Virchow et al. 2019). Therefore, the efficacy of tiotropium on improving baseline lung function suggests the involvement of M1 and M3 muscarinic receptors and by extent an

enhanced release of ACh from cholinergic nerves in the excessive bronchospasm seen in asthma. Although ACh can also be released by non-neuronal cells such as epithelial cells, the contribution of non-neuronal ACh in mediating bronchoconstriction has yet to be proven (Kummer and Krasteva-Christ 2014).

To date, no allergen challenge study has tested the efficacy of a LAMA on the LAR in atopic asthmatics. However, the Respiratory Pharmacology group has recently showed that tiotropium blocked the LAR in a preclinical rat OVA model displaying both EAR and LAR-like reactions responsive to gold standard therapies (Raemdonck et al. 2012). This finding was replicated by another group, with tiotropium preventing the LAR in a guinea pig OVA model (Smit et al. 2014). Furthermore, the LAR was also lost under ketamine/xylazine anaesthesia in the rat OVA model, confirming the involvement of a central nerve reflex (Raemdonck et al. 2012). Therefore, it was hypothesised that the LAR could be driven by a cholinergic nerve reflex.

1.5.4.2 Evidence of airway sensory nerves activation in the LAR

If a cholinergic reflex is responsible for driving the LAR, this also implies that allergen challenge stimulates the afferent arm of the reflex. Accordingly, several animal studies have shown that inflammatory mediators released upon allergen challenge can stimulate airway sensory nerves (Undem and Taylor-Clark 2014). In addition, allergen challenge has been associated with an increase in spontaneous coughing measured over 24 h in mild atopic asthmatics (Satia et al. 2019). This observation further supports the hypothesis that allergen challenge leads to a cascade of events that activates airway sensory nerves and, after integration in the CNS, enhances airway reflexes such as cough and reflex bronchospasm.

As previously mentioned, mast cells are found in close proximity to airway nerve fibres (Undem and Taylor-Clark 2014). In animal studies, early mediators released from mast cells upon allergen challenge depolarised vagal sensory neurons isolated from guinea pigs or rabbits *in vitro* (Undem, Hubbard, and Weinreich 1993; Greene et al. 1988) and increased airway Aδ- and C-fibres excitability *ex vivo* and *in vivo* in rats and guinea pigs, for a duration that outlasts the EAR time span (Riccio, Myers, and Undem 1996; G. Zhang et al. 2008). In other studies, isolated mediators known to be released from mast cells such as LTD_4, PGD_2 and proteases increased the excitability of airway Aδ- and C-fibres (Taylor-Clark, Nassenstein, and Undem 2008; L. Y. Lee and Morton 1993; G. Zhang et al. 2008; Kwong and Lee 2002; 2005; Q. Gu and Lee 2006; Kwong et al. 2010). Given that mast cells are amongst the first cells activated by allergen challenge, the crosstalk between mast cells and airway sensory nerves could be involved in initiating the LAR.

So far it remains unclear which cells and mediators could be responsible for activating airway sensory nerves after allergen challenge and leading to a cholinergic reflex. This thesis aimed to study possible activators of airway sensory nerves involved in the LAR, which could help to decipher new mechanisms causing the LAR and by extent airflow limitation in atopic asthmatics.

1.6 The role of TRP channels in the late asthmatic response

Many inflammatory mediators released upon allergen challenge, such as CysLTs, PGD_2 or proteases released by mast cells activate metabotropic GPCRs. To generate a sufficient depolarisation to elicit action potential firing in neurons, GPCRs have been shown to activate ion channels such as transient receptor potential (TRP) channels, with their opening causing an influx of cations depolarising the neuron membrane (Belvisi and Birrell 2017; Mazzone and Undem 2016). In particular, numerous studies have shown that TRPV1, TRPA1 and TRPV4 channels can be stimulated by inflammatory mediators and activate airway sensory nerves, leading to airway reflexes such as cough (Belvisi and Birrell 2017). Interestingly, the Respiratory Pharmacology group found that ruthenium red, a non-selective TRP channels blocker, prevented the LAR in the rat OVA model (Raemdonck et al. 2012). Therefore, TRP channels, and in particular TRPV1, TRPA1 and TRPV4, are promising candidates for eliciting airway sensory nerves activation and a cholinergic reflex bronchospasm in the LAR. Current evidence of their involvement in the LAR will be discussed in this section.

1.6.1 Definition of TRP channels

TRP channels are a superfamily of 28 channels divided into 7 families based on sequence homology, namely: TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin), TRPC (canonical), TRPML (mucolipin) and TRPN (no mechanoreceptor potential C). TRPs are non-selective cation ion channels constituted by a tetramer of TRP proteins, with each monomer exhibiting intracellular N and C termini and 6 transmembrane segments S1-S6, with S5-S6 segments forming the central ionic pore. TRP channels are polymodal: an unprecedented variety of physical and chemical stimuli seems to activate them and these channels are therefore considered as cellular sensors. As such, endogenous lipid mediators, temperature, mechanical stress, oxidative stress and exogenous compounds were found to stimulate TRP channels (S. F. Pedersen, Owsianik, and Nilius 2005). GPCRs activated by inflammatory mediators were also shown to indirectly stimulate TRP channels, by inducing intracellular signalling either leading to TRP channels opening (“receptor operated gating”) or increasing TRP channels probability to open to their cognate stimuli (“sensitisation”) (Darby et al. 2016).

1.6.2 TRPV1 channels

TRPV1 channels have been amongst the most widely studied channels on airway sensory nerves. These channels are highly expressed on airway sensory neurons, as attested by single-cell RT-PCR (Kwong et al. 2008; Nassenstein et al. 2010; Wortley et al. 2016). Their activation fires airway C-fibres and subsets of Aδ-fibres in anaesthetised guinea pigs (Adcock et al. 2014). TRPV1 channels are activated by the exogenous compound capsaicin (pepper extract) and capsaicin is one of the most potent tussive agents in human (Laude, Higgins, and Morice 1993). In the body, TRPV1 channels appear to be stimulated by noxious heat >42^{0}C, lipid eicosanoid mediators derived from arachidonic acid such as 12-(S)-HPETE, 15-(S)-HPETE, 5-(S)-HETE, and LTB_4, or upon GPCR signalling elicited by bradykinin, PGE_2 or agonists of protease-activated receptors 2 (PAR2) (S. F. Pedersen, Owsianik, and Nilius 2005; Grace et al. 2012; Amadesi et al. 2004).

Increased *TRPV1* gene expression has been found in asthmatic patients as well as genetic variants associated with a lower risk of childhood asthma (Belvisi and Birrell 2017). However, the role of TRPV1 in asthma has been controverted in preclinical models. Mouse models of allergic airway disease have displayed either protective, non-significant or detrimental effect of TRPV1 on airway eosinophilia and AHR and the effect depends on the model and strain used (Caceres et al. 2009; Mori et al. 2011; Delescluse, Mace, and Adcock 2012; Rehman et al. 2013; Baker et al. 2016; Tränkner et al. 2014). In the Respiratory Pharmacology group, two distinct TRPV1 antagonists did not significantly reduce the LAR in the rat OVA model (Raemdonck et al. 2012; Baker et al. 2016). In contrast, a TRPV1 antagonist reduced airway inflammation and AHR in a mouse OVA model with ozone exposure mimicking pollution-induced asthma exacerbation (J. Li et al. 2019). In human, allergen challenge increased capsaicin-evoked cough responses in patients with mild atopic asthma (Satia et al. 2019) and patients with stable asthma exhibited exaggerated capsaicin-evoked cough responses at baseline compared to healthy volunteers, with nonatopic asthmatics exhibiting the highest responses (Satia et al. 2017). However, it is unknown whether an increased sensitivity to TRPV1 stimulation is indicative of a role for TRPV1 in asthma pathophysiology (Belvisi et al. 2017).

1.6.3 TRPA1 channels

TRPA1 channels are also highly expressed on airway sensory neurons, as attested by single-cell RT-PCR (Nassenstein et al. 2008; Wortley et al. 2016) and TRPA1 activation induces the firing of airway C-fibres but not airway Aδ-fibres in anaesthetised guinea pigs (Adcock et al. 2014). TRPA1 are also found expressed in non-neuronal cells such as airway smooth muscle, epithelial and mast cells (Belvisi and Birrell 2017). TRPA1 seems to be directly activated by reactive electrophile species, notably endogenous products found in inflammatory states such as reactive oxygen species (ROS), eicosanoids peroxidation products or endogenous aldehydes (Belvisi and Birrell 2017). GPCRs activation by inflammatory mediators such as PGE_2, bradykinin or PAR2 agonists has also been shown to sensitise TRPA1 channels (Grace et al. 2012; Dai et al. 2007).

TRPA1 is distinctly activated by a wide range of electrophile exogenous compounds found in environmental pollutants, such as acrolein from cigarette smoke, toluene diisocyanate from manufacturing processes, diesel exhaust particles or ozone (Andrè et al. 2008; Taylor-Clark et al. 2009; Taylor-Clark and Undem 2010; R. K. Robinson et al. 2018). Mice deficient in TRPA1 exhibited reduced AHR to toluene diisocyanate or hypochlorite (Taylor-Clark et al. 2009; Devos et al. 2016; Hox et al. 2013). Activation of TRPA1 could therefore play an important role in eliciting symptoms provoked by environmental irritants in nonatopic asthma.

In contrast, the role of TRPA1 has been less studied in atopic asthma, although a genetic variant of *TRPA1* was found associated with an increased risk of childhood asthma (Gallo et al. 2017). The Respiratory Pharmacology group showed that a TRPA1 antagonist diminished the LAR in the rat OVA model previously described to involve a cholinergic reflex (Raemdonck et al. 2012). In contrast, TRPA1 deficiency had no effect on AHR and eosinophilia in a mouse model provoked by HDM (Baker et al. 2016). These findings suggest that TRPA1 could play a role in triggering nerve-mediated symptoms in certain endotypes of asthma.

1.6.4 TRPV4 channels

TRPV4 is another TRP channel with a high degree of homology to TRPV1, however TRPV4 has been less studied in airway reflexes. TRPV4 is also a cation ion channel, but with a higher permeability than TRPV1 or TRPA1 for large divalent ions such as calcium or magnesium (Deng et al. 2018).

1.6.4.1 Expression of TRPV4 channels

TRPV4 channels are expressed in a wider range of tissues and cells than TRPA1 or TRPV1 (Belvisi and Birrell 2017). In the respiratory tract, TRPV4 is highly expressed by structural cells, with its expression found in human primary cells including airway epithelial cells, airway smooth muscle cells, fibroblasts, alveolar macrophages and endothelial cells (Baxter et al. 2014; D. F. Alvarez et al. 2006; Jia et al. 2004; McAlexander et al. 2014; Rahaman et al. 2014; Fantozzi et al. 2003). In animal studies, TRPV4 was found expressed in vagal ganglia tissue (L. Zhang et al. 2004; Brierley et al. 2008; Ni et al. 2006), however it does not seem to be expressed on airway sensory neurons themselves but rather on cells that surround the neurons (Bonvini et al. 2016).

1.6.4.2 Activation of TRPV4 channels

TRPV4 is a polymodal channel shown to integrate many different physical and chemical stimuli. The channel is constitutively active at body temperature, with an activation in the 24-40^{0}C range (Güler et al. 2002; H. Watanabe, Vriens, et al. 2002). It was originally discovered as an osmosensor activated by hypotonicity (Liedtke et al. 2000; Strotmann et al. 2000; Liedtke and Friedman 2003) and was shown to respond to shear stress (Köhler et al. 2006; Gevaert et al. 2007). In addition, TRPV4 is activated by the arachidonic acid metabolites 5'6'EET, 8'9'EET derived from the cytochrome epoxygenase (CYPe) pathway (H. Watanabe et al. 2003; Darby et al. 2016). It is however unclear how TRPV4 is activated by these stimuli and whether these endogenous mediators directly bind to the channel. Recent cryo-EM analysis found densities in the pore region of the channel that could correspond to these endogenous lipids binding to TRPV4 (Deng et al. 2018). In fact, hypotonicity may indirectly stimulate TRPV4 via the generation of 5'6'EET (Vriens et al. 2004).

Independently from unknown endogenous ligands binding to the channel, TRPV4 has been shown to be sensitised or activated by inflammatory mediators activating GPCRs such as histamine, 5-HT and proteases activating PAR2, via mechanisms including protein kinases A and C (PKA, PKC) and tyrosine kinase mediated phosphorylation of the channel (Cenac et al. 2010; Grant et al. 2007; Grace et al. 2014).

In the laboratory, the exogenous agonists 4αPDD and GSK1016790A are used to study TRPV4 and directly bind to the channel, with GSK1016790A reported to be more selective and potent than 4αPDD (Thorneloe et al. 2008; Alexander et al. 2013; H. Watanabe, Davis, et al. 2002).

1.6.4.3 TRPV4 activates airway sensory nerves via the release of ATP

Recently, the Respiratory Pharmacology group has demonstrated that TRPV4 activates airway sensory neurons. Exogenous 4αPDD and GSK1016790A agonists as well as hypotonicity depolarised vagal nerves isolated from guinea pigs, mice and human, with the signal being blocked by TRPV4 antagonists or genetic deficiency. *In vivo*, the TRPV4 agonist GSK1016790A and hypotonicity provoked a sustained firing of airway sensory Aδ-fibres, but not C-fibres, in anaesthetised guinea pigs (Bonvini et al. 2016).

Contrary to TRPV1 and TRPA1, TRPV4 channels seem to indirectly activate airway sensory neurons, via inducing the release of adenosine triphosphate (ATP) through ATP-permeable pannexin-1 channels (Panx1) and the released ATP activating purinergic P2X3-P2X2/3 receptors on the neurons as shown in (**Figure 1-7**). Indeed, TRPV4-induced vagus signal was blocked by the P2X3-P2X2/3 antagonist AF-353 and by Panx1 deficiency (Bonvini et al. 2016). This notion will be further explained in the following section.

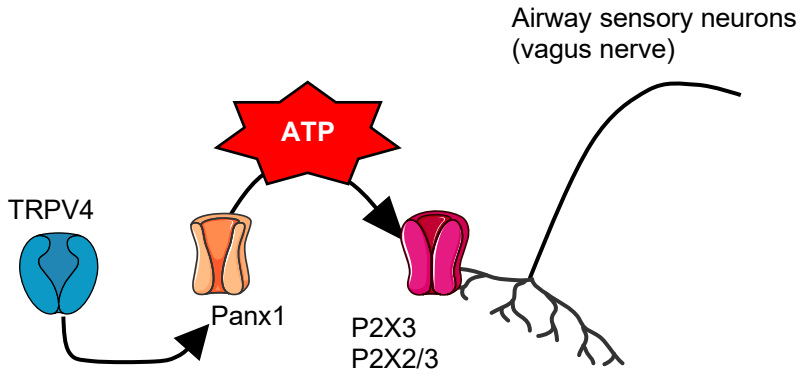


Figure 1-7 The TRPV4-ATP-P2X3 nerve axis

TRPV4 activation triggers the opening of Panx1 channels, which release ATP into the extracellular milieu. The released ATP activates P2X3-P2X2/3 receptors expressed on airway sensory neurons, inducing the firing of airway sensory Aδ-fibres comprised within the vagus nerve.

It is intriguing and counterintuitive that TRPV4, a cation-permeable channel, would require a secondary mediator to activate neurons. However, recent evidence shed light on this notion with the findings that TRPV4 may not be expressed on neurons but rather on cells that

surround the neurons (Alexander et al. 2013; Rajasekhar et al. 2015). Previous study from the group indeed found scarce gene expression of *Trpv4* in single isolated airway sensory neurons from guinea pigs, contrary to TRPA1 and TRPV1 (Bonvini et al. 2016). It is unclear which cells would express TRPV4 and release ATP activating airway neurons. Since ATP is very labile and degraded within minutes by endogenous ectonucleotidases (ATPases) (Gorman, Feigl, and Buffington 2007), it can be hypothesised that cells expressing TRPV4 and responsible for activating airway neurons must be in close vicinity to airway nerve terminals.

1.6.4.4 ATP activates airway sensory nerves via P2X3-P2X2/3 receptors

Adenosine triphosphate (ATP) is a known activator of airway sensory nerves in animal studies (Canning et al. 2004; Kollarik et al. 2003; Undem et al. 2004). Although ATP is a ubiquitous intracellular molecule which can be released by cellular damage, it can also be released in a controlled manner through the opening of pannexins and connexins channels. Extracellular ATP activates purinergic P2 receptors, with ATP preferentially binding to P2X ionotropic than P2Y metabotropic receptors.

P2X receptors are a family of ion channels widely expressed in the body that comprises seven members P2X1-P2X7 which function as homotrimers or heterotrimers (Burnstock 2007; North 2002). P2X3 receptors have gained important attention as activators of sensory neurons. Concurrently, P2X3 expression seems to be restricted to sensory neurons (Burnstock 2007; North 2002). P2X3 are permeable to sodium and calcium and assemble as P2X3 homotrimers (P2X3/P2X3/P2X3) or as P2X2/3 heterotrimers with calcium-permeable P2X2 receptors (P2X3/P2X3/P2X2) (North 2002). In the airways, single-cell RT-PCR studies found that nodose neurons express both *P2x2* and *P2x3* while jugular neurons only seem to express *P2x3* (Kwong et al. 2008; Nassenstein et al. 2010; Bonvini et al. 2016). Interestingly, a same concentration of ATP strongly induces the firing of nodose-derived fibres but only a sparse discharge of jugular-derived fibres in guinea pigs (Canning et al. 2004; Undem et al. 2004; Weigand, Ford, and Undem 2012). Therefore, it has been hypothesised that ATP activates airway sensory neurons mostly via P2X2/3 heterotrimers compared to P2X3 homomers. This was confirmed in studies using genetically-modified mice and pharmacological compounds in rats and cell lines (Cockayne et al. 2000; 2005; Zhong et al. 2001; Lewis et al. 1995).

Interestingly, TRPV4 was shown to only fire airway Aδ- but not C-fibres in anaesthetised guinea pigs, and to induce calcium flux in airway nodose- but not jugular-derived guinea pig neurons *in vitro* (Bonvini et al. 2016). This finding corroborates the notion that TRPV4 activates airway sensory neurons via the release of ATP that stimulates P2X2/3 heterotrimers on airway neurons.

1.6.4.5 The role of the TRPV4-ATP-P2X3 nerve axis in asthma

Currently, only two publications have investigated TRPV4 in atopic asthma, concluding a role for TRPV4 in airway remodelling (Gombedza et al. 2017) or a dispensable role in airway inflammation and AHR (Palaniyandi et al. 2019) using mouse models of allergic airway disease. In addition, TRPV4 activation provoked airway smooth muscle contraction in human and guinea pig large airways *in vitro* via inducing the release of CysLTs from mast cells (McAlexander et al. 2014; Bonvini et al. 2020).

Although the role of the TRPV4-P2X3 nerve axis has never been studied in asthma, it has been shown to provoke cough, another airway reflex, in guinea pigs (Bonvini et al. 2016). Furthermore, the P2X3-P2X2/3 antagonist AF-219 (gefapixant) was associated with an unprecedented 75% reduction in objective cough frequency in patients with refractory chronic cough (Abdulqawi et al. 2015), suggesting that therapies targeting the ATP-P2X3 pathway are promising for diseases involving airway reflexes. Interestingly, aerosolised ATP provokes bronchoconstriction in asthmatic patients (Basoglu et al. 2005) and ATP-induced bronchoconstriction was blocked by the P2X3-P2X2/3 antagonist DT-0111 in conscious guinea pigs (Pelleg et al. 2019). Therefore, activation of airway sensory nerves via the ATP-P2X3 axis could also be involved in triggering reflex bronchospasm in asthma.

Protease-activated receptors 2 (PAR2) have been shown to stimulate TRPV4 and to elicit a sensory nerve signal in the context of pain (Sipe et al. 2008; Grant et al. 2007; Poole et al. 2013; Zhao et al. 2014; 2015; Grace et al. 2014). In asthma, PAR2 activating proteases such as tryptase are found elevated in the airways of asthmatic patients at baseline (Aubier et al. 2016). Importantly, allergen challenge is associated with a rapid increase in tryptase levels in BAL fluid (Sedgwick et al. 1991; S. E. Wenzel, Fowler, and Schwartz 1988) and a tryptase inhibitor reduced the LAR but not the EAR in atopic asthmatics (Krishna et al. 2001). ATP, the downstream mediator of TRPV4, has also been found elevated in the BAL of atopic asthmatics after allergen challenge (Idzko et al. 2007).

Since both TRPV4 downstream mediator and upstream activators are elevated after allergen challenge, it can be hypothesised that TRPV4 could be one of the drivers of the LAR, via activating airway sensory nerves via P2X3-P2X2/3 receptors and triggering reflex bronchospasm. This thesis will be focused on investigating the role of TRPV4 in driving a nerve reflex leading to the LAR.

1.7 Animal models of the late asthmatic response

To investigate the role of TRPV4 in driving a nerve reflex in the LAR, a model mimicking the EAR and LAR and responsive to relevant asthma therapies was needed. Most allergic airway disease models are set-up in mice, followed by rats and to a lesser extent guinea pigs. In these animals, atopic asthma does not naturally occur and is thus artificially provoked, most commonly using ovalbumin (OVA) or house dust mite (HDM) although other allergens are being increasingly used such as *Alternaria* and *Aspergillus* fungi. Typically, animals are firstly systemically or topically sensitised to the allergen, following which topical allergen challenge provokes features of atopic asthma including eosinophilic inflammation, AHR, allergen-induced bronchoconstriction and airway remodelling (Zosky and Sly 2007; Aun et al. 2018). These features are highly dependent on the species, strain, allergen, adjuvant, route of administration and frequency of the challenges.

1.7.1 Models according to the allergen

1.7.1.1 Ovalbumin (OVA) models

Ovalbumin models are amongst the most commonly used models of allergic airway disease and have been established in mice, rats and guinea pigs, using ovalbumin protein (OVA) from chicken egg as an allergen. Animals are typically sensitised to OVA administered systemically (i.p. or s.c.) 2 to 3 times over 2 weeks, following which animals are topically challenged with OVA (aerosol or i.n.). to induce airway responses. Most OVA models are acute (Aun et al. 2018).

OVA models are set-up with systemic sensitisation because topical sensitisation has not been successful. Indeed, repeated airway challenges with OVA has been associated with the development of tolerance, an observation also justifying the fact that most OVA models are acute (Swirski et al. 2002; van Hove et al. 2007). Systemic sensitisation to OVA usually requires an adjuvant (most commonly aluminium hydroxide derivatives) that primes the immune system to develop a T_H2 response against OVA. Although the mechanism is unclear, it has been hypothesised that aluminium-containing adjuvants promote monocytes differentiation into APCs and the uptake of the allergen by APCs, with a T_H2 polarised response (Lambrecht et al. 2009). In sensitised animals, acute OVA challenge characteristically triggers T_H2-driven eosinophilic inflammation in the airways (Stevenson and Birrell 2011). Depending on the species used, OVA models can display nonspecific AHR and/or allergen-induced bronchoconstriction (Aun et al. 2018; Stevenson and Birrell 2011).

However, this model has been highly criticised over the fact that OVA is not a clinically relevant allergen, nor does systemic sensitisation with an adjuvant mimic how asthmatic patients are likely sensitised. OVA models are also criticised for their acute nature and lack of airway remodelling, with changes quickly resolving in absence of the allergen and repeated challenges inducing tolerance, contrary to that observed in asthmatic patients (Stevenson and Birrell 2011). For these reasons, other models were set-up with allergens encountered in the clinic such as house dust mite (HDM).

1.7.1.2 House dust mite (HDM) models

House dust mite (HDM) models use a mixture of allergens derived from *Dermatophagoides pteronyssimus* (*Der p*) and *Dermatophagoides farinae* (*Der f*), to which an important proportion of atopic asthmatics are sensitised (Stevenson and Birrell 2011). HDM models have been mostly described in mice and to a lesser extent in rats and guinea pigs, with many protocols ranging from acute to chronic challenges.

Contrary to OVA, HDM allergens are not inert: HDM possess intrinsic protease activity and immunogenicity and airway exposure to HDM (i.n.) induces sensitisation without an adjuvant (Aun et al. 2018). In addition, repeated topical HDM challenges provoke severe airway inflammation associated with airway remodelling, features which persist after the last HDM exposure (Johnson et al. 2004). It was the use of HDM models that led to the discovery of the involvement of the epithelial-ILC2-innate cells axis in airway responses (Lambrecht, Hammad, and Fahy 2019). Nonetheless, this important participation of innate immune cells has also led to question the relevance of chronic HDM models to study allergic mechanisms (Stevenson and Birrell 2011). Furthermore, there are currently no published studies reporting an EAR and LAR-like bronchospasm to HDM challenge. Therefore, it was not possible to use an HDM model in this thesis.

1.7.2 Models according to the species

1.7.2.1 Mouse OVA models

Few groups have reported an LAR in mouse OVA models (Zosky and Sly 2007; Stevenson and Belvisi 2008), with four main models characterised (Meyts et al. 2008; Nabe et al. 2005; Cieslewicz et al. 1999; Baker et al. 2017).

However, the IgE-mast cell axis does not seem to play an important role in mouse models. Indeed, mouse models have shown an LAR in the absence of EAR (Meyts et al. 2008) or in presence of an EAR but with different mechanisms than observed in human. In mice, acute

bronchospasm to OVA has been attributed to the release of 5-HT from mast cells activating 5-HT2 receptors on parasympathetic neurons and inducing ACh release (Cyphert et al. 2009; Weigand et al. 2009). Furthermore, in models reporting an EAR, the LAR did not seem to be dependent on mast cells nor IgE (Nabe et al. 2013; Baker et al. 2017), contrasting with the finding that omalizumab reduces the LAR in atopic asthmatics (Fahy et al. 1997; Trischler et al. 2017).

In addition, the relevance of inflammatory events leading to allergen-induced bronchospasm in mouse models has been questioned. A time-course comparison found that lymphocyte recruitment peaked earlier in atopic asthmatics than in a mouse OVA model (Lommatzsch et al. 2006). Furthermore, mouse OVA models were used to demonstrate the efficacy of anti-IL-5 and anti-IL-13 therapies on the LAR, which contrasts with the results found in atopic asthmatics (Cieslewicz et al. 1999; Taube et al. 2002; Leckie et al. 2000; Scheerens et al. 2014), although these findings depend on the model used (Meyts et al. 2008; Nabe et al. 2011).

Importantly, the LAR response was not blocked by anaesthesia in several mouse models (Meyts et al. 2008; Cieslewicz et al. 1999; Baker et al. 2017), nor by the LAMAs tiotropium or glycopyrrolate, nor by a TRPA1 antagonist (Baker et al. 2017). These data suggest that the LAR displayed in mouse models is not driven by a nerve reflex. Therefore, mouse models were not chosen for this thesis.

1.7.2.2 Guinea pig OVA models

Contrary to mice or rats, guinea pig airways present numerous similarities with humans in terms of airway innervation, airway smooth muscle reactivity and mast cell mediators (Zosky and Sly 2007; Canning and Chou 2008; West et al. 2015). Studies have reported OVA guinea pig models displaying an EAR blocked by LTRAs and antihistamines (Riley et al. 2013) and an LAR blocked by ICS, LABA, LAMA (Smit et al. 2014).

However, the LAR measured in guinea pig OVA models is highly variable between groups (Stevenson and Belvisi 2008). More importantly, guinea pigs exhibit high airway reactivity to nonspecific irritants, with eosinophil-like cells (heterophils) present at baseline in the airways. This is considered to be an important caveat for studying airflow limitation induced specifically by allergic mechanisms. In addition, local axon reflexes involving tachykinins play an important role in provoking bronchoconstriction in guinea pigs and it is unclear whether these peripheral reflexes play an important role in human (Stevenson and Belvisi 2008; Zosky and Sly 2007; Adner et al. 2020).

1.7.2.3 Rat OVA models

The LAR has been consistently modelled in the rat species in several groups. These rat OVA models display both an EAR and LAR with airway eosinophilia, but not all models display an associated AHR (Stevenson and Belvisi 2008).

Inflammatory processes have been less studied in rats, however Brown Norway rats are known to be genetically predisposed to elicit strong T_H2 and IgE-dependent responses (Stevenson and Belvisi 2008). Eosinophilic inflammation was reported to peak later than the LAR in the rat OVA model used in the Respiratory Pharmacology group, as it has been reported for human (S. L. Underwood et al. 2002; Gauvreau, Watson, and O'Byrne 1999; Lommatzsch et al. 2006).

Contrary to mice, mast cells and IgE were shown to play a role in rat OVA models. Indeed, inhibiting IgE production blocked the EAR and LAR in one model (Nonaka et al. 2000) and mast cell-deficient rats failed to exhibit an LAR in another (S. Liu, Chihara, and Maeyama 2005). Nonetheless, mast cell biology differs between rats and humans. In rats, the EAR depends on 5-HT and LTD_4 while 5-HT is not important in humans (Hele et al. 2001; Wohlsen et al. 2003; Roquet et al. 1997; Davis et al. 2009). Concurrently, rat airway smooth muscle is less responsive to bronchoconstrictors such as histamine and LTD_4 compared to human (Lulich and Paterson 1980; Seehase et al. 2011).

In spite of these species differences, the rat OVA model used in the Respiratory Pharmacology group displays both an EAR, LAR and eosinophilic inflammation that respond to clinically relevant compounds: the EAR is blocked by a combination of 5-HT, histamine, LTD_4 antagonists and the LAR by the gold standard therapies ICS and LABA. More importantly, the LAR in this model was abolished by anaesthesia and by the LAMA tiotropium, finding which suggested that a central cholinergic reflex drives the LAR. The LAR was also reduced by the pan-TRP antagonist ruthenium red and by a TRPA1 antagonist, further supporting the involvement of airway sensory nerves in the response (Raemdonck et al. 2012). Therefore, this model was chosen to study the involvement of TRPV4 in driving a nerve reflex in the LAR.

1.8 Thesis Plan

The mechanisms driving airflow limitation in atopic asthma remain poorly understood. In the laboratory, inhalation of a high dose of allergen by atopic asthmatics can lead to a prolonged episode of airway narrowing named the LAR, providing a model to study mechanisms driving allergen-specific airflow limitation (Gauvreau, El-Gammal, and O'Byrne 2015). The LAR has been viewed as the result of eosinophil and/or basophil influx secreting mediators such as CysLTs in the airways, however an anti-IL-5 therapy reducing eosinophil numbers was found ineffective against the LAR and not all patients respond to LTRAs (Leckie et al. 2000; Szefler et al. 2005). Recent preclinical work from the Respiratory Pharmacology group suggested that airway nerves could play a role in the LAR, with allergen challenge resulting in activation of airway sensory nerves and a cholinergic reflex bronchospasm (Raemdonck et al. 2012). Studying this nerve reflex could help to decipher new mechanisms driving the LAR and to develop therapies for atopic asthma targeting other pathways than type-2 inflammation. The overall aim of this thesis was to build upon this hypothesis, by investigating the TRPV4 channel as a potential candidate for driving a nerve reflex leading to the LAR.

1.8.1 Hypothesis

Activation of the TRPV4-P2X3 axis by allergen challenge leads to the activation of airway sensory nerves and, after integration in the CNS, provokes a reflex cholinergic bronchospasm resulting in the LAR.

1.8.2 Aims

- Validate if the TRPV4-P2X3 nerve axis can activate airway sensory nerves in naïve rats: using selective TRPV4 and P2X3 agonists and antagonists **(Chapter 3)**.
- Explore the role of the TRPV4-P2X3 axis in the LAR: by evaluating ATP levels and testing selective TRPV4 and P2X3 antagonists in the rat OVA model **(Chapter 4)**.
- Investigate if the TRPV4-P2X3 nerve axis can provoke a cholinergic reflex bronchospasm in naïve rats: by testing the LAMA tiotropium against TRPV4-induced bronchospasm **(Chapter 5)**.
- Investigate how allergen challenge can lead to the activation of the TRPV4-P2X3 nerve axis, by studying PAR2 receptors as potential activators of the TRPV4-P2X3 axis: via evaluating PAR2 upregulation in the rat OVA model, testing the effect of PAR2 activation on airway sensory nerves in naïve rats and testing selective PAR2 antagonists in the rat OVA model **(Chapter 6)**.

2 General methods

This chapter describes the general methodology for experimental protocols used throughout this thesis. Specific protocols and statistical analysis are detailed in individual result chapters.

2.1 Animals

All animal work was approved by Imperial College London Ethics Review Committee and conformed strictly to the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986.

Animals were housed in individually ventilated cages under specific pathogen-free conditions at 21^{0}C with food and water *ad libitum*. Animals were acclimatised for at least 7 days prior to any experimental procedure.

2.1.1.1 Rats

Male Brown Norway rats (200 - 250 g) were purchased from Charles River Laboratories (Germany).

2.1.1.2 Mice

Stock breeders of C57BL/6J (*Par2*$^{+/+}$) and B6.Cg-*F2rl1*^{tm1Mslb}/J (*Par2*$^{-/-}$) mice (Schmidlin et al. 2002) were purchased from Jackson Laboratory (USA). Homozygous *Par2*$^{-/-}$ were viable, fertile and bred on a C57BL/6J background at Imperial College London. *Par2*$^{+/+}$ C57BL/6J mice were bred alongside in separate cages to be used as littermate controls. Male *Par2*$^{-/-}$ and *Par2*$^{+/+}$ mice were used for the experiments and genotyped to confirm gene disruption.

2.2 Electrophysiological measurements

To investigate whether the TRPV4 axis activates airway sensory nerves in naïve rats, three complementary electrophysiological techniques were used.

2.2.1 *In vitro* isolated vagus preparation

The isolated vagus preparation evaluates the ability of drug solutions to elicit compound depolarisation of an isolated vagus nerve trunk *in vitro*. The vagus nerve was used as it is the main nerve innervating the airways. Furthermore, as neuronal proteins are translated in the cell body and transported through axons to nerve terminals, it is hypothesised that the vagus trunk can be used as a surrogate to study the effect of drugs on receptors expressed by vagus terminals. This technique is amenable to relatively high-throughput pharmacology studies using a minimal number of animals and allows translational studies using human tissue. This technique also enables to study the effect of drugs on vagus depolarisation independently from influences of bronchoconstriction, mucus secretion or vascular leakage that can indirectly activate the vagus nerve (Bergren 1997; Bonham et al. 1996; Kappagoda and Ravi 1989; Widdicombe 2003; Kollarik et al. 2003), in addition of avoiding pharmacokinetic considerations.

2.2.1.1 Isolated vagus set-up

Male Brown Norway rats (200 – 300 g) and *Par2*$^{-/-}$, *Par2*$^{+/+}$ male C57BL/6J mice were euthanised via overdose of pentobarbitone (200 mg/kg, i.p.). Skin, upper muscles, connective tissues and rib cage were removed to expose the two vagal trunks running from the vagal ganglia at the base of the skull, alongside the trachea and to the heart. The portions of the two vagal nerves running alongside the trachea were dissected free and cleaned of connective tissue. Nerves were desheathed from their epineurium, cut into 15 mm-long segments and placed into Krebs-Henseleit (KH) solution (mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5 and glucose 5.6) at 37°C and bubbled with 95% O_2 / 5% CO_2 to maintain oxygenation and pH:7.4 for the rest of the experiment.

The dissected vagus nerve segments were mounted into a “grease-gap” recording chamber in a custom-made Perspex box as described in (Birrell et al. 2002) and shown in (**Figure 2-1**). Each segment rested in a longitudinal channel without tension, with the two ends electrically and chemically isolated using petroleum jelly. One end contacted the recording electrode and was perfused at a rate of 2 ml/min either with KH solution alone or KH containing test drugs, maintained at 37°C and bubbled with 95% O_2 / 5% CO_2. The other end was placed onto a reference electrode and kept in KH solution throughout the experiment. Both electrodes were

glass pipettes filled with Ag/AgCl pellets (Mere 2 Flexible reference electrodes, WPI, UK) and connected to a DAM50 differential amplifier (WPI, UK) which measured, amplified (x10 gain) and filtered (high cut-off: 1 KHz, sample rate 5 Hz) the potential difference between the two electrodes. This was converted into a digital signal and analysed using LabScribe 3 software (iWorx).

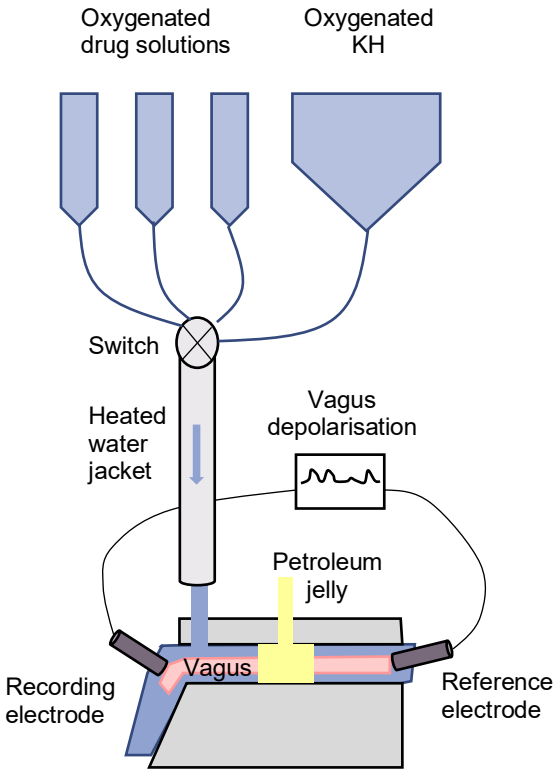


Figure 2-1 Isolated vagus nerve preparation

Isolated vagus segments were drawn into a "grease-gap" recording chamber and each end of the nerve chemically and electrically isolated with petroleum jelly. The left side of the nerve contacted the recording electrode and was perfused with heated and oxygenated KH or drug solution. The right side of the nerve bathed in KH solution and contacted the reference electrode. Drug incubation can depolarise the left side of the vagus nerve, creating a potential difference compared to the right side. The potential difference between the two electrodes was amplified and recorded as vagus depolarisation.

The vagus preparation indirectly measures the ability of a drug to provoke the depolarisation of neurons present within the vagus trunk. The potential difference measured by Ag/AgCl electrodes is generated by the movement of Cl^- ions in aqueous solution between the AgCl electrode, the KH solution and the axon cytosol. If a drug activates receptors on the axon membrane and provokes an influx of cations such as Na^+ or Ca^{2+} drawn from the extracellular KH solution into the axon cytosol, this leaves an excess of Cl^- ions in the KH solution. The Cl^-

left in excess then react with Ag^+ in the recording electrode. As no similar Cl^- movement operates at the reference electrode, this creates a positive potential difference between the two electrodes recorded as a depolarisation (mV). As the recording electrode is placed on a cross-section of the whole vagus nerve, the measured potential difference reflects the sum of the depolarisations of each axon that have been stimulated within the vagus trunk, named "compound depolarisation". After depolarisation, the axon membrane repolarises to resting potential as K^+ exit the cytosol via potassium channels. The K^+ ions left in excess in the KH solution combine with Cl^- ions from the recording electrode and the potential difference returns to baseline.

It should be noted that recording vagus depolarisation does not ascertain whether a drug activates the neurons, i.e. provokes action potential firing. Indeed, as the recording electrode is placed at the same position where the nerve is stimulated by the drug, it is unknown whether the recorded depolarisation is due to action potential firing that propagated through the axon. In addition, the recorded depolarisation can be caused by subthreshold Na^+ or Ca^{2+} neuronal movements that are insufficient to generate action potential firing, and non-excitable cells such as endothelial cells or accessory cells present within the vagus nerve could also be stimulated and contribute to cations movements. It should also be noted that the vagus nerve supplies other organs than the airways and this preparation does not specifically assess airway sensory neurons. A signal elicited on the vagus trunk may also not reflect a signal on the nerve terminals (Spaulding and Burgess 2017). Nonetheless, studies from the laboratory found that vagus nerve depolarisation most commonly translates into airway sensory nerve activation and *in vivo* responses (Grace et al. 2012; Bonvini et al. 2016; Nasra and Belvisi 2009). In addition, other techniques were used to complement the vagus preparation to specifically assess the activation of airway sensory nerve terminals: the tracheal nerve preparation and the single fibre preparation.

2.2.1.2 Isolated vagus experiments

Drugs were diluted in KH solution and added using 1:1000 dilutions according to protocols detailed in individual result chapters.

2.2.1.3 Agonist concentration-response

Agonists concentration-responses were conducted in the same piece of nerve (paired data). The nerve segment was incubated with each concentration of the agonist or its vehicle in a randomised order to avoid de/sensitisation bias. Each application lasted for 2 min and 10 min-long washes were carried out between each concentrations. Following characterisation, a submaximal concentration of agonist could then be taken forward for antagonist experiments.

Only one concentration-response of up to five concentration points could be tested on each piece of nerve from each animal.

2.2.1.4 Agonist/antagonist response

Agonist responses were compared in absence and presence of an antagonist in the same piece of nerve (paired data). A single concentration of agonist and antagonist was used per piece of nerve. The nerve was firstly incubated with the agonist for 2 min then washed until the response returned to baseline. This was repeated to ensure reproducibility of the response and washed. Thereafter, the nerve was incubated with the antagonist for 10 min, before reapplying the agonist in presence of the antagonist for 2 min. The nerve was then washed to re-establish baseline and re-incubated with the agonist for 2 min to ascertain recovery of the response and nerve viability. Only one concentration of antagonist was tested on each piece of nerve from each animal.

2.2.1.5 Data analysis

Data were analysed as the peak depolarisation (mV) from baseline for each stimulation. For antagonist experiments, the peak depolarisations elicited by the first two agonist stimulations were averaged and compared to the depolarisation elicited by the agonist in presence of the antagonist. The percentage inhibition was calculated for each antagonist with the formula:

$$\% \text{ Inhibition} = \frac{Agonist\ mean\ depolarisation - Agonist\ depolarisation\ in\ presence\ of\ antagonist}{Agonist\ mean\ depolarisation} \times 100$$

Statistical analysis was performed on the raw data expressed as depolarisation (mV). Data were analysed using paired Students t-tests. The compound depolarisation reflects the summation of all depolarisations elicited on each axon within the vagus nerve. It was assumed that each axon can depolarise and therefore the vagus depolarisation can be modelled by a binomial distribution (discrete distribution). As there is an important number of axons within the nerve bundle, this can be approximated by a normal distribution (continuous distribution).

2.2.2 *In vitro* isolated tracheal nerve preparation

The isolated tracheal nerve preparation was used to assess the effect of a compound on the activation of airway nerve terminals. This preparation measures the ability of drug solutions to elicit compound action potential firing from the tracheal nerve endings of the recurrent laryngeal nerve (RLN) *in vitro*. Large airways record the highest density of innervation within the respiratory system (Jeffery and Reid 1973; A. Laitinen 1985; Mortola, Sant'ambrogio, and Clement 1975; Sant'Ambrogio et al. 1978) and most of the trachea is innervated by the RLN (Ricco et al. 1996; Yamamoto et al. 1994; Canning et al. 2004; Liebermann-Meffert et al. 1999), highlighting the relevance of studying the RLN in the investigation of airway reflexes. In addition, evaluating the activity of lower airway terminals derived from the vagus nerve *in vitro* would require to isolate the lungs *en bloc* and to maintain correct lung perfusion, oxygenation and delivery of the drugs within the lungs (Kollarik et al. 2003). This tracheal nerve preparation is therefore advantageous as it enables to study the activation of upper airway nerve terminals in a controlled setting, independently from influences of mechanical factors or delivery considerations.

2.2.2.1 Isolated tracheal nerve set-up

Male Brown Norway rats (200 – 300 g) were euthanised via overdose of pentobarbitone (200 mg/kg, i.p.). Skin, upper muscles, connective tissue and rib cage were removed to expose the upper airways. The larynx, trachea, lungs and their innervation from the vagal ganglia down to the mediastinal region were isolated *en bloc*. The preparation was cleaned of connective tissue. At the end of the dissection remained the larynx, trachea and main bronchi connected to their respective nerve branches, comprising the vagal ganglia, SLN, RLN and adjacent vagus nerve in a similar manner to (Riccio, Myers, and Undem 1996). Tissues were incubated with KH solution (mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5 and glucose 5.6) at 37°C and bubbled with 95% O_2 / 5% CO_2 to maintain oxygenation and pH:7.4.

The RLN was cut caudal to the main vagus nerve, preserving its connections to the trachea, and was placed into an independent bath perfused with KH solution at a rate of 5 ml/min. The airways lumen (larynx, trachea, main bronchi) were placed into a separate bath perfused with KH or drug solutions at a rate of 2 ml/min entering via the larynx, going through the tracheal lumen and exiting via the left and right bronchi onto a waste trap, without coming into contact with the bath containing the RLN. The RLN was desheathed and the sectioned end placed onto a recording suction electrode. A reference electrode was positioned on the surrounding fascia (**Figure 2-2**). Electrodes were glass pipettes filled with Ag/AgCl pellets (Shielded Bipolar Suction Electrode, A-M systems, USA). The potential difference between the two electrodes

was recorded via connection to a headstage (NL100AK Headstage, Digitimer, UK) and the signal was pre-amplified (NL104A-AC Preamplifier, Digitimer, UK), amplified (x10,000, NL106-AC/DC Amplifier, Digitimer, UK) and filtered (300 Hz to 3000 Hz, NL125/6-Band-Pass Filter, Digitimer, UK). Noise was further removed using a Humbug (Digitimer, UK) and the output was converted to a digital signal (Micro1401-4 data acquisition unit, CED, UK) and analysed with Spike 2 software (CED, UK).

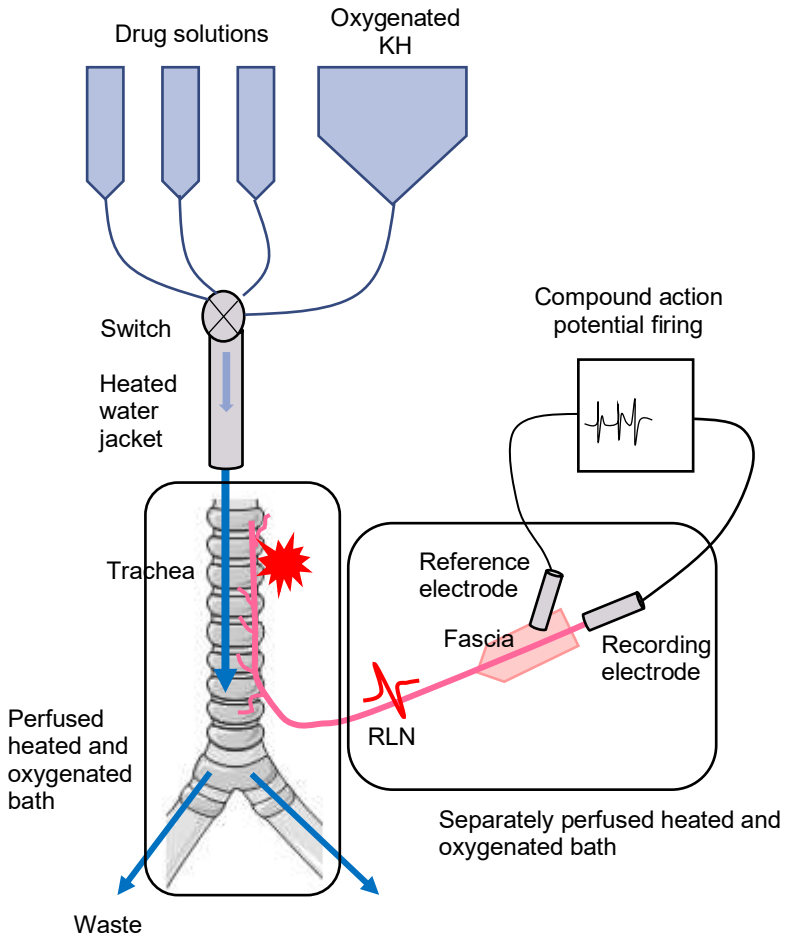


Figure 2-2 Isolated tracheal nerve preparation

The trachea and main bronchi were isolated from a naïve rat and placed in a bath. The RLN still attached to the trachea was placed in a separate bath. The RLN was desheathed and placed onto a suction recording electrode, with a reference electrode positioned on the surrounding fascia. Tissues were constantly perfused with oxygenated and heated KH solution. Drug solutions were only perfused within the tracheal lumen and did not come into contact with the bath containing the RLN. If a drug induced sufficient depolarisation of the RLN terminals in the trachea, this triggered an action potential that propagated through the axon to the recording electrode, recorded as compound action potential firing.

The tracheal nerve set-up utilizes the same type of Ag/AgCl electrodes as in the isolated vagus preparation. Therefore, electrophysiological principles described in **2.2.1.1** apply to this preparation. The difference is that drugs are perfused within the airway lumen and do not come into contact with the RLN trunk, which is in a separately perfused compartment where it connects to the recording electrode. This means that a signal recorded by the electrode would be caused by the drug acting on RLN terminals within the trachea and inducing the firing of action potentials that propagated up to the recording electrode. Therefore, this tracheal nerve preparation measures the ability of drug solutions to elicit action potential firing of airway nerve terminals of the recurrent laryngeal nerve. As the recording electrode is placed on the whole nerve section, this is named “compound action potential firing”.

2.2.2.2 Tracheal nerve experiments

Drugs were diluted in KH solution and added using 1:1000 dilutions according to protocols detailed in individual result chapters. Protocol layouts were similar to isolated vagus experiments described in **2.2.1.2**, using longer incubation times to allow the drug to diffuse through the tracheal layer onto the nerve endings. Details of the protocols are specified in individual result chapters.

2.2.2.3 Data analysis

Data were analysed as nerve mass activity (NMA) (μV.sec). NMA corresponds to the integration of all rectified compound action potential firing events over the period of stimulation, normalised to baseline activity for the same period of time.

2.2.3 *In vivo* single fibre preparation

The single fibre preparation was used to assess the effect of a compound on the activation of airway sensory nerve terminals. This preparation measures the ability of aerosolised drugs to elicit action potential firing of the terminals of a single airway sensory nerve fibre within the vagus nerve in an anaesthetised animal. This preparation also enables to study whether an *in vitro* effect on vagus depolarisation translates into nerve fibre activation *in vivo*.

2.2.3.1 Single fibre set-up

The single fibre preparation was set-up as described in (Adcock et al. 2003). Male Brown Norway rats (300 – 350 g) were anaesthetised with urethane (1.5 g/kg, i.p.) supplemented as required. The trachea was cannulated and the animals artificially ventilated (tidal volume of 9 ml/kg, 60 breaths/min) to maintain blood gases and pH using a small animal ventilator (Ugo Basile, Italy). Body temperature was monitored with a rectal thermometer and maintained at 37ºC with a heated blanket (Harvard Apparatus, UK). The right carotid artery was cannulated to monitor systemic arterial blood pressure and heart rate using a pressure transducer (SP 844 MEMSCAP, USA) linked to Spike 2 software data acquisition system (CED, UK) via a Micro1401 interface (CED, UK). Tracheal pressure was also continuously recorded with a pressure transducer (SP 844 MEMSCAP, USA) connected to a side arm of the tracheal cannula and linked to Spike 2 software via the Micro1401 interface. The right jugular vein was cannulated for the injection of drugs. Animals were paralysed with pancuronium bromide, initially administered at a dose of 0.10 mg/kg, i.v., followed every 20 min with 0.05 mg/kg, i.v. to maintain paralysis. This ensured that skeletal muscle twitches would not interfere with the sensitive nerve recordings. Because of this, depth of anaesthesia was continuously assessed by monitoring heart rate and blood pressure.

A cervical incision was made to locate the cervical vagal nerves, which were dissected free and cut at their central end (bilateral cervical vagotomy) to prevent vagal reflexes. The skin and muscles around the incision were sutured to a metal ring to form a well which was filled with mineral oil. Using a binocular microscope, the left vagus nerve was desheathed and filaments of nerve were teased free until a single fibre was obtained and placed onto a recording electrode. A reference electrode was positioned on the surrounding fascia. Electrodes were bipolar Teflon-coated platinum electrodes (WPI, UK). The potential difference between the two electrodes was recorded via connection to a pre-amplifier headstage (NL100K, Digitimer, UK). The signal was amplified (NL104, Digitimer, UK), filtered (NL125, Digitimer, UK) and passed through a Humbug noise reducer (AutoMate Scientific, USA) and the output converted to a digital signal through the Micro1401 interface and fed to the Spike 2

software package. The signal was also monitored using a digital storage oscilloscope (Tektronix DPO 2012) and fed through an audio amplifier to a loudspeaker.

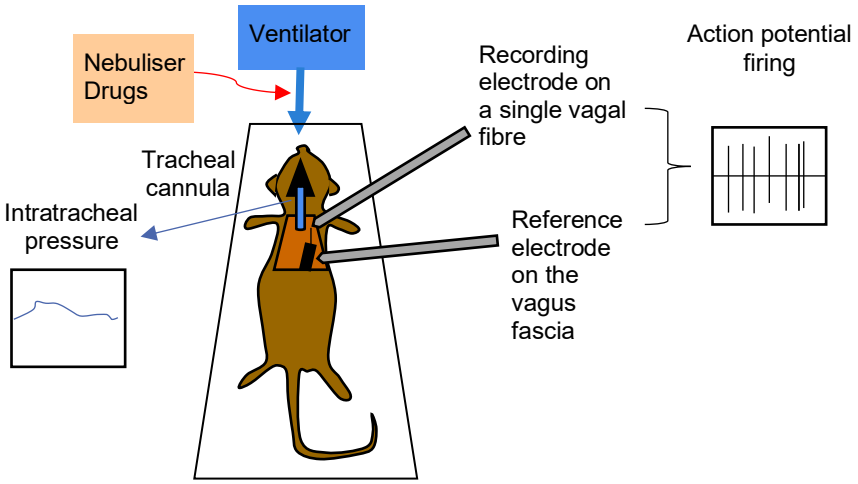


Figure 2-3 Single fibre preparation

A rat was anaesthetised, paralysed and cervical vagal nerves cut at their central end to prevent vagal reflexes. The left vagus nerve was desheathed and teased until a single fibre was obtained and placed onto a recording electrode. A reference electrode was positioned on the surrounding fascia. Drugs were nebulised via a tracheal cannula and intratracheal pressure recorded via a pressure transducer connected to the tracheal cannula. Action potential firing of the single fibre and intratracheal pressure changes were continuously measured.

Using the same principle as described in **2.2.2.1** for the tracheal nerve preparation, this technique records action potential firing. However, the difference is that it specifically records single action potential units. The teased nerve fibre was identified as belonging to one of the three main groups of airway sensory nerve terminals: SARs, RARs (Aδ-fibres) or C-fibres as described in **1.5.1.1** and using criteria stated in (Adcock et al. 2003). In this thesis, only RARs Aδ-fibres were assessed, as TRPV4 was shown to activate Aδ-fibres but not C-fibres in guinea pigs (Bonvini et al. 2016). These Aδ-fibres were identified by their spontaneous activity with a rhythmical respirator pattern, their rapidly adapting response to pulmonary hyperinflation/deflation and their response to an aerosol of citric acid (0.3 M). This Aδ-fibre selection process also ensured that the nerve fibre was originating from the airways, given that it responded to pulmonary mechanical and chemical stimulation. Conduction velocity is normally assessed by electrical stimulation to confirm the nature of the fibre (Adcock et al. 2003). Because it was the first time that the preparation was set-up in the rat and the available electrical stimulation equipment was not scaled for rats, conduction velocity could not be assessed in this thesis.

After surgery, animals were allowed to stabilise for at least 30 min prior to any experiment. Compound solutions were nebulised using an Aerogen Nebuliser (Buxco Nebulizer Control-5, Data Science International, USA) which was connected to the ventilator so that aerosolised compounds entered the airways via the tracheal cannula. This allowed to study the effect of a drug on eliciting action potential firing of the airway terminals of an airway sensory vagal fibre.

2.2.3.2 Single fibre experiments

Following identification of an airway sensory Aδ-fibre (RAR), baseline activity was recorded for at least 2 min. Thereafter, compounds were nebulised following specific protocols described in individual result chapters.

2.2.3.3 Data analysis

Data were analysed as the frequency of action potential firing events over the period of stimulation above baseline activity in (impulses/sec). No statistical analysis was performed due to the limited number of experiments conducted in this thesis.

2.3 Rat model of allergic airway disease

The role of the TRPV4 axis was investigated in the LAR using a rat model of allergic airway disease induced by OVA.

2.3.1 Protocol of the rat OVA model

Male Brown Norway rats (200 – 250 g) were sensitised on days D0, D14 and D21 with OVA (100 µg OVA per rat, 1 ml, i.p.) administered with Alum™ adjuvant (20 mg aluminium hydroxide and 20 mg magnesium hydroxide per rat, diluted 1:1 with saline) or the vehicle SAL (Alum™ diluted 1:1 with saline). On D28, rats were challenged with OVA aerosol (1% OVA w/v in saline) for 30 min in a Perspex chamber (**Figure 2-4**).

To monitor the LAR response, conscious unrestrained rats were placed 1 h after challenge in flow whole body plethysmography (FWBP) chambers (Buxco Electronics, USA) for a period of 5 h, from 1 h to 6 h post-challenge. To assess airway inflammation, rats were euthanised with an overdose of pentobarbitone (200 mg/kg, i.p.) 24 h post-challenge and bronchoalveolar lavage (BAL) was collected to evaluate inflammatory cell counts. To evaluate inflammatory mediators, rats were euthanised at timepoints specified in individual result chapters.

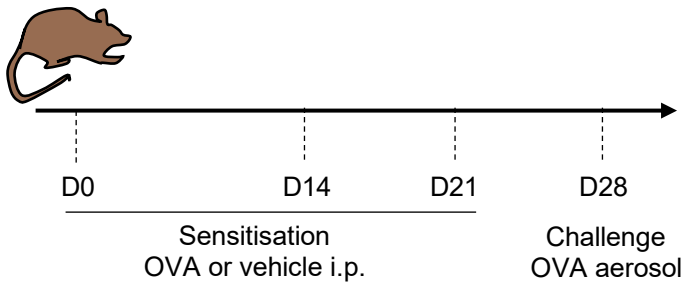


Figure 2-4 Protocol of the rat OVA model

Previous studies characterised the model by comparing the following combinations (sensitisation/challenge): SAL/SAL, SAL/OVA, OVA/SAL, OVA/OVA. On D28, only OVA-sensitised rats challenged with OVA exhibited an immediate bronchoconstriction resolving within 30 min (EAR), followed by a prolonged bronchospasm lasting from 1 h to 6 h post-challenge (LAR) accompanied by airway inflammation (S. L. Underwood et al. 2002; Raemdonck et al. 2012). SAL-sensitised rats challenged with SAL or OVA did not show such responses, showing that OVA challenge itself does not elicit an innate response. OVA-sensitised rats only presented these responses when challenged with OVA but not SAL, ensuring that the response was specific to the OVA rather than non-specific hyperreactivity.

In this thesis, only two groups were used as controls to ensure that observed responses were due to a specific response to OVA. Study groups are specified in individual result chapters.

2.3.2 Flow whole body plethysmography (FWBP)

To evaluate the LAR provoked by OVA challenge or the respiratory response to compound inhalation, flow whole body plethysmography (FWBP) was used in conscious unrestrained rats.

2.3.2.1 Flow whole body plethysmography set-up

FWBP comprises a chamber (box) attached to a pneumotachograph that measures the flow of air coming in and out of the box (**Figure 2-5 A**). This flow is provoked by changes in box pressure that are elicited by the animal breathing, as described by (Lomask 2006) and represented in (**Figure 2-5 B**).

- During inspiration, inspiratory muscles contract, causing the chest to expand and drawing air from the box through the nose to the thorax. When entering the nasal cavity and the airways, the air is heated and humidified through a process called conditioning and changes from ~21°C, 50% humidity to 37°C, 100% humidity. Due to expansion and conditioning, the inhaled air occupies a more important volume than it did outside the animal's body. This elevates the pressure inside the box, resulting in an outward box flow measured by the pneumotachograph (**Figure 2-5 C**).
- At the end of inspiration, equilibrium is reached and the flow resolves to zero (**Figure 2-5 D**).
- At the beginning of expiration, chest recoils and compresses air within the thorax. Before passing through the nasal cavity, the air volume is therefore reduced without change in its conditioning, decreasing box pressure and inducing a peak of inward box flow ("thoracic displacement flow") (**Figure 2-5 E**).
- Thereafter, air is exhaled through the nasal cavity and reduces in temperature, also contributing to induce box flow ("nasal flow") (**Figure 2-5 F**). There is a lag between the moment where the air is compressed within the chest and the moment where it passes through the nasal cavity, reflected by a lag between thoracic displacement flow and nasal flow.

These changes in box flow elicited by the animal breathing are plotted as a "box waveform" (**Figure 2-5 G**). The parameters of this waveform can vary during bronchoconstriction provoked by OVA challenge or compound inhalation.

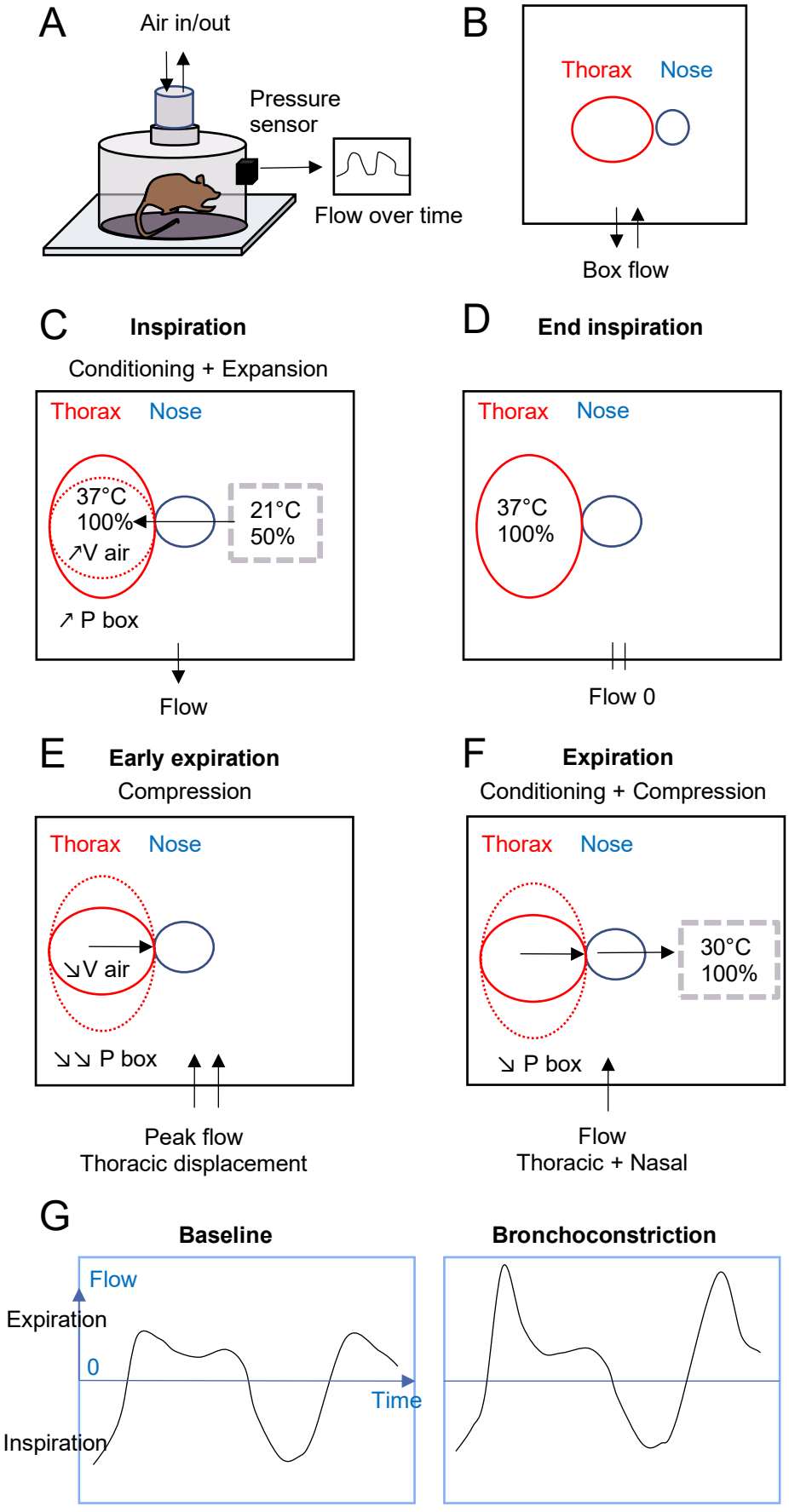


Figure 2-5 Flow whole body plethysmography (legend on next page)

Figure 2-5: Flow whole body plethysmography

(A) Representation of the FWBP chamber. **(B)** Simplified schematic of the rat body cavities involved in creating pressure changes in the FWBP chamber and resulting in box flow. **(C, D, E, F)** Relationship between air movements within the FWBP chamber, the rat thoracic and nasal cavities and the box flow during the respiratory cycle. %: percentage humidity. ↗V air: increase in air volume, ↗P box: increase in FWBP chamber pressure. **(G)** Representation of the box waveflow at baseline and during bronchoconstriction, graph adapted from (Lomask 2006). The box waveflow is obtained by plotting the differential pressure measured by the pneumotachograph inside the FWBP wall over time.

2.3.2.2 Penh parameter

To analyse the respiratory response to OVA challenge or to compounds, the composite and dimensionless Penh parameter can be calculated from the box waveflow as the following:

$$\text{Penh} = \frac{\text{PEF}}{\text{PIF}} \text{ X } \left(\frac{\text{Te} - \text{Rt}}{\text{Rt}}\right)$$

PEF: Peak Expiratory Flow
PIF: Peak Inspiratory Flow
Te: Total expiratory time
Rt; Time corresponding to 65% of expiratory flow total area

Bronchoconstriction is predominant during expiration. If airway resistance increases, for instance during the LAR, two phenomenon arise affecting Penh parameter, as represented in (**Figure 2-6**):

- Airways are contracted, therefore the air is further compressed within the thorax during early expiration and the peak box flow increases, reflected by an elevation in PEF.
- The air takes a longer time to be expelled from the chest cavity, reflected by an increase in the lag between thoracic and nasal flows and therefore an elevation in Te and reduction in Rt.

As a result, Penh variation can reflect the difference between thoracic and nasal flow and increases during bronchoconstriction (Lomask 2006; Vaickus et al. 2010).

The validity of the Penh parameter to measure airway resistance has been criticised for three main reasons (Lundblad et al. 2007; J. Bates et al. 2004; J. H. T. Bates and Irvin 2003). First, the flow waveform can be influenced by changes in temperature and humidity, as well as animal movements within the box. However, the Penh parameter mostly assesses parameters dependent on the early expiration, which are minimally dependent on conditioning, i.e. temperature and humidity. Second, a lag between thoracic and nasal flows could be caused

by an alteration in the breathing pattern rather than bronchoconstriction, although decreasing respiratory rate with an anaesthetic has not been systematically associated with changes in Penh (Hamelmann et al. 1997). Third, nasal obstruction can cause an increase in Penh, which can be problematic in rodents which are obligate nose breathers (Nakaya et al. 2006). However, it was not possible to use alternative techniques to measure bronchoconstriction in this thesis. The purpose of this work was to study the LAR in the rat OVA model, which is lost under anaesthesia (Raemdonck et al. 2012), therefore it was not an option to use invasive measurements requiring anaesthesia to measure specific airway resistance. Furthermore, the LAR is monitored over a 5-hour period, a duration for which it was not ethically acceptable to restrain rats for techniques such as double-chamber plethysmography or flow oscillation measurement. In addition, studies have demonstrated a correlation between Penh and specific measures of lung resistance upon methacholine challenge (Hamelmann et al. 1997; Verheijden et al. 2014; Bergren 2001). In this thesis, temporal, audible and visual signs of respiratory distress correlated with Penh during the LAR response. As no other non-invasive, conscious and unrestrained technique are currently known to assess airway resistance, Penh measured by FWBP was used in this thesis.

2.3.2.3 Data analysis

Penh parameter was calculated in-line by Buxco Finepoint Software (Buxco Electronics, USA). To assess the LAR response in the rat OVA model, average Penh was calculated every 10 min by the software over the 5-hour recording period. Average Penh was then plotted over time to estimate Penh area under curve (AUC). Statistical analyses were performed on Penh AUCs data.

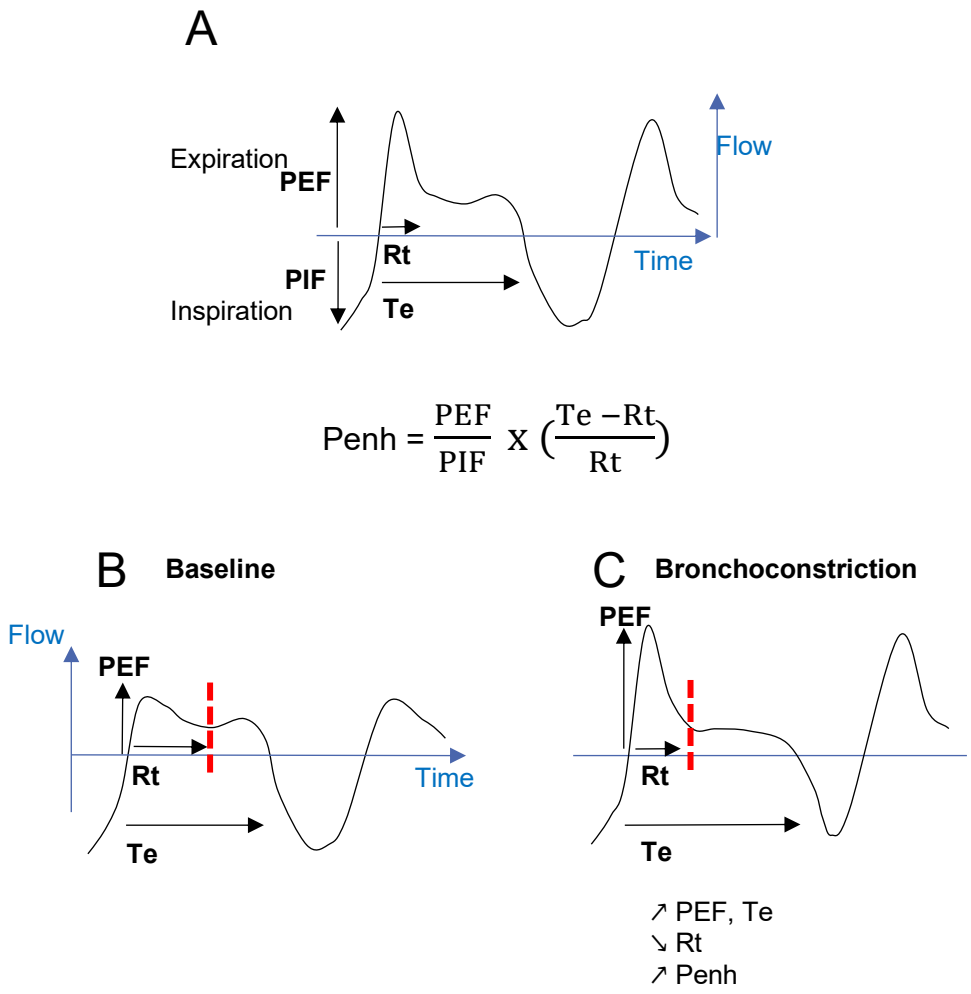


Figure 2-6 The Penh parameter

(A) Representation of the parameters determined from FWBP waveflow to calculate Penh with the formula written below the graph. **(B, C)** Representation of the variation of these parameters between baseline and bronchoconstriction states. PEF: Peak Expiratory Flow; PIF: Peak Inspiratory Flow; Rt: time corresponding to 65% of expiratory flow total area; Te: Total expiratory time.

2.3.3 Sample harvesting and processing

To evaluate airway inflammatory cell recruitment and measure mediators in the rat OVA model, rats were euthanised with an overdose of pentobarbitone (200 mg/kg, i.p.) at appropriate timepoints specified in individual result chapters. Rats were exsanguinated by cardiac puncture and their tracheas cannulated to retrieve bronchoalveolar lavage (BAL) as followed:

2.3.3.1 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluid was obtained by perfusing airways with 3 ml of medium (RPMI 1640 Glutamax, ThermoFisher Scientific, USA) at room temperature (RT), left to equilibrate for 30 sec in the body cavity before being aspirated. This procedure was repeated once and the 6 ml of lavage was combined in a single tube and stored on ice for further processing. The BAL was centrifuged (1398 g, 10 min, 4°C) to separate cells from supernatant. The supernatant was aliquoted and stored at -80°C until further analysis.

2.3.3.2 BAL differential cell counts

BAL cell pellets were reconstituted in medium (RPMI 1640 Glutamax, ThermoFisher Scientific, USA) and total leukocytes number counted with an automated Sysmex XP-300 cell counter (Sysmex Ltd., UK). For differential cell counts, BAL fluid cells were mounted onto slides using a Cytospin centrifuge (807 g, 5 min, RT) (Shandon, Runcorn, UK). Slides were stained with a modified Wright-Giemsa stain (ACCUSTAIN, Sigma-Aldrich, USA) using a Hema-tek 2000 automated slide stainer (Ames Co., Elkhart, USA). Differential counting was manually performed by counting 200 cells per slide under a light microscope (X40 magnification) and determining the percentage of eosinophils, neutrophils and monocytes/macrophages according to standard morphological criteria. The same blinded operator performed the entirety of the cell counts for consistency.

2.4 Mediator assays

2.4.1 ATP levels in BAL

ATP levels were measured in BAL fluid using the ATPlite 1-step Luminescence kit (Perkin Elmer, USA). Briefly, 100 µl of BAL supernatant were mixed with 100 µl of substrate solution in a 96-well plate and incubated for 10 min at RT on an orbital shaker protected from light. ATP standards were used as a control of the reaction. Emitted light was measured using a luminometer (Tecan Spark, Switzerland). The lower detection limit corresponded to 1 nM of ATP standard.

This assay estimates the concentration of extracellular ATP in samples via a luciferin/luciferase reaction. Luciferase converts luciferin into oxyluciferin by consuming ATP and emitting light (photons) following the reaction:

$$\text{ATP + D-Luciferin + } O_2 \xrightarrow[Mg2+]{Luciferase} \text{Oxyluciferin + AMP + PPi + } CO_2 \text{ + Photon (light)}$$

Since the reagents are in excess, the amount of light emitted is proportional to the amount of ATP in the sample. Data were analysed as raw luminescence in (photons/sec) subtracted from blank reading.

2.4.2 Tryptase-like activity in BAL

Tryptase-like activity was measured in BAL fluid using the Mast Cell Degranulation Assay Kit as per manufacturer protocol (IMM001, Merck Millipore, USA). Briefly, 180 µl of BAL supernatant were mixed with 20 µl of substrate solution in a 96-well plate and incubated for 2 h at 37°C protected from light. Tryptase standards were used as a control of the reaction. Absorbance was measured at 405 nm and 602 nm using a spectrophotometer after 2 h incubation (BiotekPowerWave XS Plate reader, USA).

This assay estimates tryptase-like enzymatic activity via cleavage of the preferential tryptase substrate tosyl-gly-pro-lys-*p*NA into free *p*NA which absorbs light at 405 nm (Schwartz et al. 1987). Preliminary tests were conducted to determine the kinetics of tryptase enzymatic reaction. At the concentration of substrate used, the enzymatic reaction reached a plateau at 2 h of incubation, timepoint therefore chosen to estimate tryptase-like activity levels. The substrate tosyl-gly-pro-lys-*p*NA is not specific to tryptase and therefore this assay potentially detects all proteases possessing a similar catalytic mechanism (“tryptase-like activity”).

Data were analysed as the absorbance at 405 nm subtracted from absorbance at 602 nm to account for potential particulate contamination of individual wells. Data were further

normalised to the absorbance of the medium used to obtain BAL samples, and to the absorbance read before adding any substrate to account for the presence of proteins absorbing at 405 nm, such as heme in BAL samples contaminated with blood.

2.5 Genotyping

Par2$^{-/-}$ and *Par2*$^{+/+}$ mice were genotyped by performing PCR on crude DNA extracted from mouse tail tips.

2.5.1.1 Crude DNA extraction

DNA extraction was performed using the Extracta DNA prep for PCR kit (Quanta Biosciences, USA). Mouse tail clips (2 mm) were incubated into 75 µl of extraction buffer and heated at 95^{0}C for 30 min. After cooling to RT, 75 µl of stabilisation buffer was added. A volume of 3 µl of each sample was taken for DNA quantification by reading absorbance of the sample at 260 and 280 nm using a spectrophotometer (BiotekPowerWave XS Plate reader, USA). DNA concentration was evaluated based on the property of nucleic acids to absorb at 260 nm. DNA purity was assessed by measuring the ratio 260/280 reflecting protein contamination. Samples were used if presenting a 260/280 ratio of $\geq$ 1.8. DNA samples concentration was adjusted to 10 ng/ml with nuclease-free H_2O.

2.5.1.2 PCR amplification

Par2 gene was amplified according to the supplier's protocol (Jackson Laboratory, USA) with minor modifications, using the Promega GoTaq Flexi DNA Polymerase kit (Promega, USA) with three types of primers (**Table 3)**. Wild-type forward (WT-Fwd) and wild-type reverse (WT-Rev) primers delimited a portion of exon present in the *Par2* gene in *Par2*$^{+/+}$ mice with a size of 385 bp. Mutant forward (Mut-Fwd) and wild-type reverse (WT-Rev) primers delimited a portion of the neomycin cassette present in *Par2* gene in *Par2*$^{-/-}$ mice with a size of 198 bp (Schmidlin et al. 2002; Hennessey and McGuire 2013). Primers were obtained from Invitrogen (ThermoFisher Scientific, USA). An amount of 5 µl (50 ng) of each DNA sample was added to the reagent mix containing (with final concentrations): 5 µl Green GoTaq Flexi Buffer (X1), 1 µl dNTPs (0.4 mM/dNTP), 2 µl $MgCl_2$ (2 mM), 0.2 µl GoTaq DNA Polymerase (0.04 U/µl), 8.8µl Nuclease-free H_2O, 1 µl of wild-type or mutant forward primers (0.4 pmol/µl) and 1 µl of wild-type reverse primers (0.4 pmol/µl) in a total volume of 25 µl. The following PCR amplification protocol was run: 1) 1 cycle: 95^{0}C 2 min; 2) 40 cycles: 95^{0}C 30 sec, 60^{0}C 30 sec; 72^{0}C 2 min; 3) 1 cycle: 75^{0}C 10 min, in a thermocycler StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, USA).

2.5.1.3 Electrophoresis separation

PCR products were separated by gel electrophoresis on a 2% agarose gel containing SafeView Nucleic Acid Stain (5 µl/100ml) (NBS Biologicals, UK) in TBE buffer (Tris/Borate/EDTA solution). A 1 kb DNA ladder (100 bp resolution) was run in parallel (Hyperladder, Bioline, UK). Gels were run for 1 h at 80 V. Gels were visualised under UV light (Odyssey Fc, LI-COR Biosciences, Germany) and images taken for analysis.

Primer	**Primer sequence 5'-3'**	**Reference/Source**
Par2 WT-Fwd	TCA AAG ACT GCT GGT GGT TG	oIMR7419 ThermoFisher Scientific, USA
Par2 Mut-Fwd	GCC AGA GGC CAC TTG TGT AG	oIMR7415 ThermoFisher Scientific, USA
Par2 WT-Rev	GGT CCA ACA GTA AGG CTG CT	oIMR7420 ThermoFisher Scientific, USA

Table 3 *Par2* genotyping primers

2.6 Measurement of airway contraction

To investigate the effect of the TRPV4 axis on provoking airway smooth muscle contraction in naïve rats, four complementary techniques were used.

2.6.1 *In vivo* measurement of Penh response using FWBP

To study the ability of a compound to provoke bronchospasm in conscious naïve rats, Penh was measured with FWBP as described in **2.3.2**.

Male Brown Norway rats (200 – 300 g) were placed in FWBP chambers (Buxco Electronics, USA) and the drug solution was directly nebulised into the FWBP chambers using a nebulizer (Buxco Electronics, USA). Specific protocols are detailed in individual result chapters. Pressure changers were continuously monitored and Penh calculated in-line using Buxco Finepointe Software (Buxco Electronics, USA). Penh data were averaged every min and plotted as Penh average over time. Statistical analysis was performed on Penh AUCs data.

2.6.2 *In vivo* measurement of intratracheal pressure changes

To study the ability of a compound to provoke local bronchospasm independently from a nerve reflex *in vivo* in naïve rats, the single fibre set-up was used as outlined in **2.2.3**.

Male Brown Norway rats (300 – 350 g) were anaesthetised, paralysed, artificially ventilated and both vagi cut at their central end as described in **2.2.3**. Drug solutions were aerosolised into the rat trachea using an Aerogen nebuliser (Buxco Nebulizer Control-5; Data Sciences International, USA) connected to the ventilator and arranged so that inspired air passed through the drug nebuliser chamber before entering the tracheal cannula. In addition of measuring single fibre activity, this preparation directly recorded intratracheal pressure changes via a pressure transducer (SP 844 MEMSCAP, USA) connected to the side of the tracheal cannula. As both rat vagi were cut at their central end, this prevented the occurrence of reflex bronchospasm and enabled to measure local contraction of the airways located below the trachea. Specific protocols are detailed in individual result chapters. Intratracheal pressure (cm H_2O) was plotted over time for analysis. No statistical analysis was performed due to the limited number of experiments conducted in this thesis.

2.6.3 *In vitro* measurement of tracheal contraction using organ bath

An organ bath preparation was used *in vitro* to evaluate the effect of compounds on upper airway smooth muscle contraction (trachea and main bronchi).

2.6.3.1 Organ bath set-up

Male Brown Norway rats (200 – 300 g) were euthanised via overdose of pentobarbitone (200 mg/kg, i.p.). Skin, upper muscles, connective tissue and rib cage were removed to expose upper airways. The trachea and main bronchi were dissected out, cleaned of connective tissue and placed into KH solution (mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5 and glucose 5.6), at 37ºC and bubbled with 95% O_2 / 5% CO_2 to maintain oxygenation and pH:7.4. Airways were maintained in KH solution containing the non-selective COX inhibitor indomethacin (10 µM) to inhibit the production of endogenous prostanoids generated by the dissection, which can interfere with smooth muscle contraction.

The trachea was opened longitudinally by cutting through the cartilage opposite to the smooth muscle layer and generating four transverse segments. The left and right main bronchi were separated and left intact. Each tracheal and bronchus segment was sutured with nylon string and tied to fixed steel hook transducers in 10 ml organ baths (Linton Instrumentation, UK) containing KH solution with or without indomethacin, at 37ºC and bubbled with 95% O_2 / 5% CO_2, as represented in (**Figure 2-7**) and described in (Buckley et al. 2011). Isometric tension changes were measured by connecting the hooks to calibrated force transducers (Grass FT-03, Grass Instruments, USA) and the signal fed to a data acquisition system (Biopac Systems MP100 workstation, USA) and analysed using AcqKnowledge software (Biopac Systems, USA).

2.6.3.2 Organ bath experiments

Tissues were left to equilibrate under a passive tension of 1 g in KH solution for 1 h, with washes every 30 min. Viability and maximal contractile response was assessed at the beginning of each experiment by incubating tissue segments with a supramaximal concentration of acetylcholine ACh (1 mM). Once tension reached a plateau, tissues were washed three times with KH solution. This process was repeated three times to ensure reproducibility of ACh response. The third ACh response was taken as a reference of the maximal tissue response. After ACh responses, tissues were left to equilibrate to baseline and passive tension reset to 1 g prior to conducting experiments. Drugs were diluted in KH solution and added manually into the bath using 1:1000 dilutions according to protocols detailed in

individual result chapters. At the end of the experiment, tissues were washed and incubated with ACh (1 mM) to confirm that tissues were still viable.

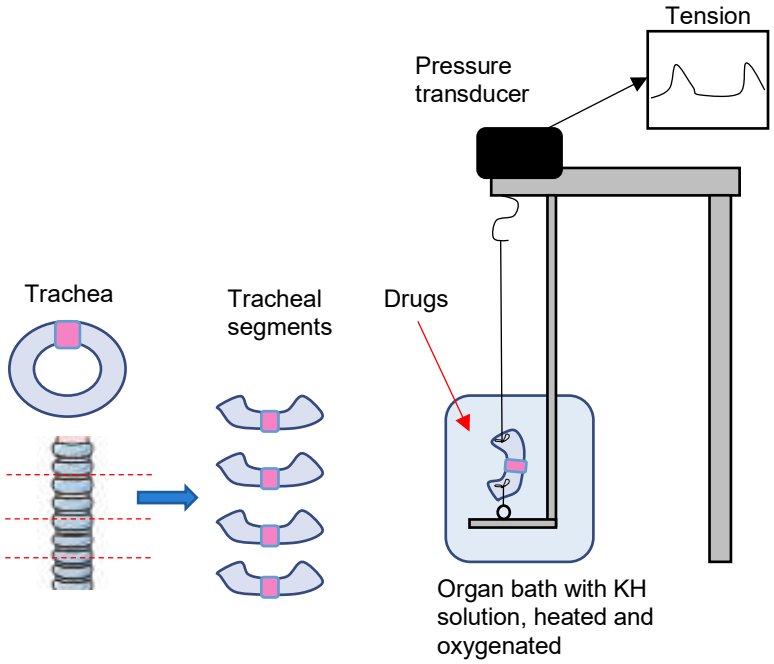


Figure 2-7 Organ bath preparation of isolated tracheas

Tracheas were isolated from naïve rats and cut into segments placed into individual organ baths, bathed in heated and oxygenated KH solution. Tissue segments were tied to a system of hooks connected to a calibrated pressure transducer that measured changes in isometric tension to assess tracheal contraction.

2.6.3.3 Analysis

Data were expressed as isometric tension (mg), which reflected tracheal smooth muscle contraction and was determined as the maximum tension of a tissue segment recorded during the drug incubation period. For statistical analysis, data were normalised to the maximal ACh contraction of each tissue segment.

2.6.4 *In vitro* imaging of small airways contraction using precision-cut lung slices (PCLS)

To evaluate the effect of compounds on small airways contraction, precision-cut lung slices (PCLS) were generated from naïve rats and contraction evaluated using light microscopy following the layout described in (**Figure 2-8**). PCLS were generated on day 0. Integrity, viability and contractility of airways were assessed on day 1 by incubating slices with ACh, in order to assign airways with a similar range of diameters and contractility between treatment groups for the experiments conducted on day 2. This technique was implemented for the first time in the laboratory, therefore thorough validation work was conducted during this thesis.

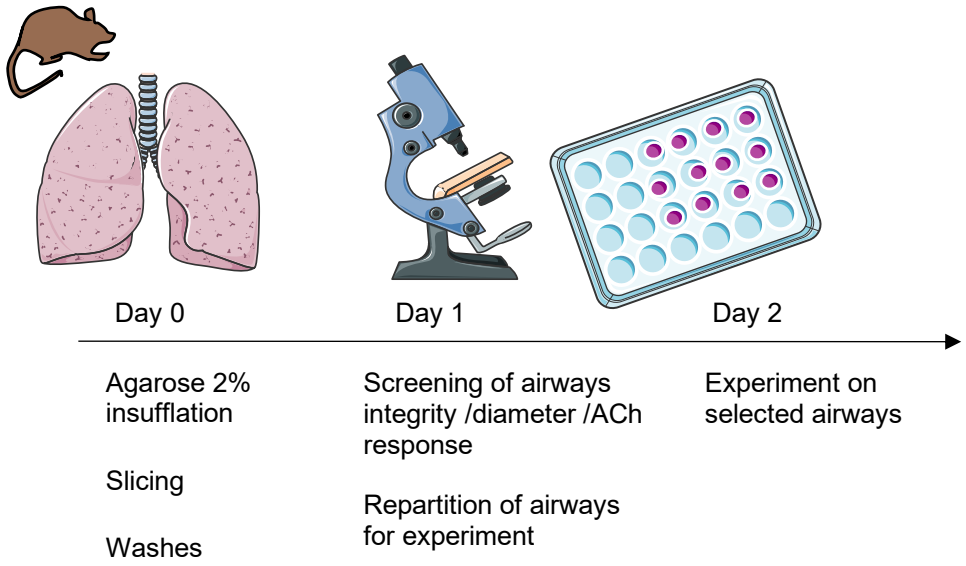


Figure 2-8 Layout of experiments measuring small airways contraction in PCLS

2.6.4.1 Day 0: PCLS generation

On day 0, male Brown Norway rats (200 – 300 g) were euthanised via overdose of pentobarbitone (200 mg/kg, i.p.). Skin, upper muscles and rib cage were removed to expose the trachea and lungs. Rats were exsanguinated by cutting the vena cava. The trachea was cannulated and lungs slowly perfused with 2% Ultra-Low Gelling Temperature Agarose prepared in HBSS with 100 units/ml penicillin - 0.1 mg/ml streptomycin kept at 37 °C (references of reagents in **Appendix**). The solution was perfused until lungs were fully inflated, corresponding to 5 ml of solution per 100 g of rat body weight. The trachea was tied off and the lungs taken *en bloc* and immerged in ice-cold HBSS with penicillin-streptomycin for 30 min on ice for the agarose to set. Once set, cylindrical lung cores were transversally cut from the left lung lobe using a tissue coring tool with a diameter of 10 mm (Harvard Apparatus, UK). Generated lung cores were longitudinally cut into PCLS using a VF-300-OZ Compresstome

(Precisionary Instruments LLC, USA), with a thickness of 250 µm. Approximately 40 slices were generated per rat. PCLS were individually plated in 24-well plates, each PCLS freely immerged in 1 ml of DMEM complete medium (DMEM high glucose medium supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 µg/ml amphotericin B, 4 mM L-glutamine, and 1% MEM non-essential aminoacids solution) at 37 ºC with 5% CO_2 in an incubator to maintain pH:7.4. Slices were washed every 30 min for 3 h to remove inflammatory mediators generated during the slicing process before letting them to rest overnight in DMEM complete medium.

2.6.4.2 PCLS live-imaging set-up

Small airways contraction was assessed using live-imaging with light microscopy. For all imaging sessions, PCLS were washed twice and incubated in 1 ml of extracellular solution (ECS) (in mM: KCl 5.4; NaCl 136; $MgCl_2$ 1; $CaCl_2$ 2.5; NaH_2PO_4 0.33; HEPES 10; glucose 10; supplemented with 10% FBS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 µg/ml amphotericin B, 4 mM L-glutamine, and 1% MEM non-essential aminoacids solution) adjusted at pH:7.4 and warmed at 37ºC. Slices were left to equilibrate in ECS for 1 h at 37ºC in a non-gassed incubator before any imaging. This buffer maintained pH equilibration without the need of a CO_2 atmosphere, which was not possible under the microscope.

Slices were imaged in their 24-well plate using brightfield microscopy with a widefield inverted microscope Zeiss Axiovert 200 (Zeiss, Germany) in a temperature-controlled chamber set at 37ºC. Slices were allowed to move freely in ECS medium, without any tension applied. Images were acquired with Zen 2 blue edition - pro imaging software (Zeiss, Germany) using a 10x objective. Each well of the 24-well plate contained a single PCLS, bearing 1 to 3 identifiable airways. Manual snapshots of up to 40 airways were simultaneously taken at specified timepoints. The same airways were tracked by following their (X; Y; Z) coordinates on the calibrated motorised stage using Sample Carrier function. Snapshots were treated using the open source processing package Fiji (ImageJ, version 2.0, (Schindelin et al. 2012)). Image files were processed in Zeiss *.czi format retaining time of acquisition and scale information. Images of the same airway recorded at different timepoints were stacked together using a custom-made macro (Stephen Rothery, Facility for Imaging by Light Microscopy (FILM), Imperial College London). As slices floated freely in the medium, airways could move between timepoints. Therefore, images were registered (aligned) using the Descriptor Based Registration Time series (2d/3d + t) plugin available in the Fiji package (Stephan Preibisch, ImageJ version 2.0, (Schindelin et al. 2012)). Airway contraction was followed by measuring the area of empty airway lumen (μm^2) at each timepoint by setting a manual threshold to distinguish levels of grey between the airway lumen and the background and analysing area

using the Analyze Particles function. Airway diameter (µm) was calculated as the Feret's diameter of delimited airways. Contraction was monitored at each timepoint by calculating the percentage closure of airway lumen with the following formula:

$$\text{Percentage closure at timepoint } t_x\ (\%): \frac{(Area\ at\ baseline - Area\ at\ timepoint\ tx)}{Area\ at\ baseline} \times 100$$

2.6.4.3 Day 1 post-isolation: PCLS airways screening

As PCLS are generated from a whole lung lobe, many different airway generations can be assessed from one animal, with important inter-airway variability (Dandurand et al. 1993; Minshall et al. 1997). It was notably observed that the smaller the airway, the more it contracts to a muscarinic agonist (C. Martin, Uhlig, and Ullrich 1996). It was therefore important to ensure that similar airways were compared between treatment groups. For this reason, airways diameter, viability, and maximum contractility were firstly screened by performing an ACh response. Because of this time-consuming process, PCLS experiments were conducted across two days, with ACh screening on day 1 and the experiment on day 2 post-isolation. Preliminary validation work ensured that airways responsive to ACh on day 1 were still responsive on day 2 with a same magnitude of the response (**Figure 2-9**). This concurred with studies in the literature showing viability of the PCLS for up to 72 h by assessing LDH release (Akram et al. 2019; Ressmeyer et al. 2006; C. Martin, Uhlig, and Ullrich 1996). This two-day process also allowed the airways to fully wash off the ACh response and restore baseline patency as shown in (**Figure 2-9**). The decision to test pharmacological compounds on day 2 was also made to reduce potential confounding effects of airway inflammation due to the PCLS isolation process. Studies from the literature have indeed reported high levels of inflammatory cytokines resulting from the slicing which decrease after 24-48 h (Behrsing et al. 2013) and PCLS metabolic activity seems to recover after 24 h (Hirn et al. 2014; Temann et al. 2017).

ACh was chosen to evaluate airways viability and contractility at a concentration (1 mM) shown to elicit supramaximal airway contraction (Bergner and Sanderson 2002a). Many PCLS studies use stable muscarinic analogues such as MCh (C. Martin, Uhlig, and Ullrich 1996; Ressmeyer et al. 2006; Neuhaus et al. 2017), however, because ACh is metabolised by esterases, the contraction is more readily reversible and this was preferred to perform experiments on the same PCLS after ACh screening.

PCLS were washed twice, equilibrated in 1 ml of ECS at 37°C for 1 h in a 24-well plate and transferred under the microscope as described in **2.6.4.2**. PCLS were screened for potential airways and their position on the 24-well plate recorded. Each airway was clearly identified with a coding system to keep track of the same airways from day 1 to day 2. Less than 10 min

after baseline images were taken, 100 µl of ACh (x11) solution was added to each well, to achieve a final concentration of ACh 1 mM. After 10 min, images of each airway were captured, with examples represented in (**Figure 2-9**). Validation work established that airway contraction to ACh plateaued at 10 min as shown in (**Figure 2-9**), similar to a published report (Bergner and Sanderson 2002a). Afterwards, PCLS were washed twice in ECS and incubated in 1 ml of warm DMEM complete medium in a 5% CO_2 incubator at 37°C.

During this incubation, the contraction to ACh was analysed and airways were selected for the experiment according to the following criteria:

- Airways presenting a transverse circular section, as the slicing can generate longitudinal cuts due to the oblique trajectory of airways.
- Intact airways, with intact parenchymal attachments, no visible tear of the PCLS and no debris within the airway lumen. Selected airways were not situated on the edge of a slice, as this area can be subjected to mechanical strain.
- Airways were morphologically identified based on the presence of an epithelial and smooth muscle lining and the proximity of a blood vessel.
- Only airways that contracted more than 50 % to ACh (1 mM) were selected. This arbitrary cut-off was chosen to assess airway viability as ACh has been shown to provoke on average 80% airway closure in PCLS (Bergner and Sanderson 2002a). This also confirmed their identification as airways, since arteries can resemble airways but dilate or do not respond to ACh (C. Martin, Uhlig, and Ullrich 1996; Perez and Sanderson 2005).

Once selected, airways were assigned to treatment groups for the pharmacological experiment on the following day according to the following criteria:

- A similar range of airway diameters between groups for each animal.
- A similar range of maximum contraction to ACh between groups for each animal.
- Approximately the same number of airways in each group and at least 3 airways per treatment group for each animal.

At the end of day 1 post-isolation, PCLS bearing selected airways were transferred using a flat spatula into a new 24-well plate for the experiment. After transfer, slices were washed and left to rest overnight in fresh DMEM complete medium in a 5% CO_2 incubator at 37°C.

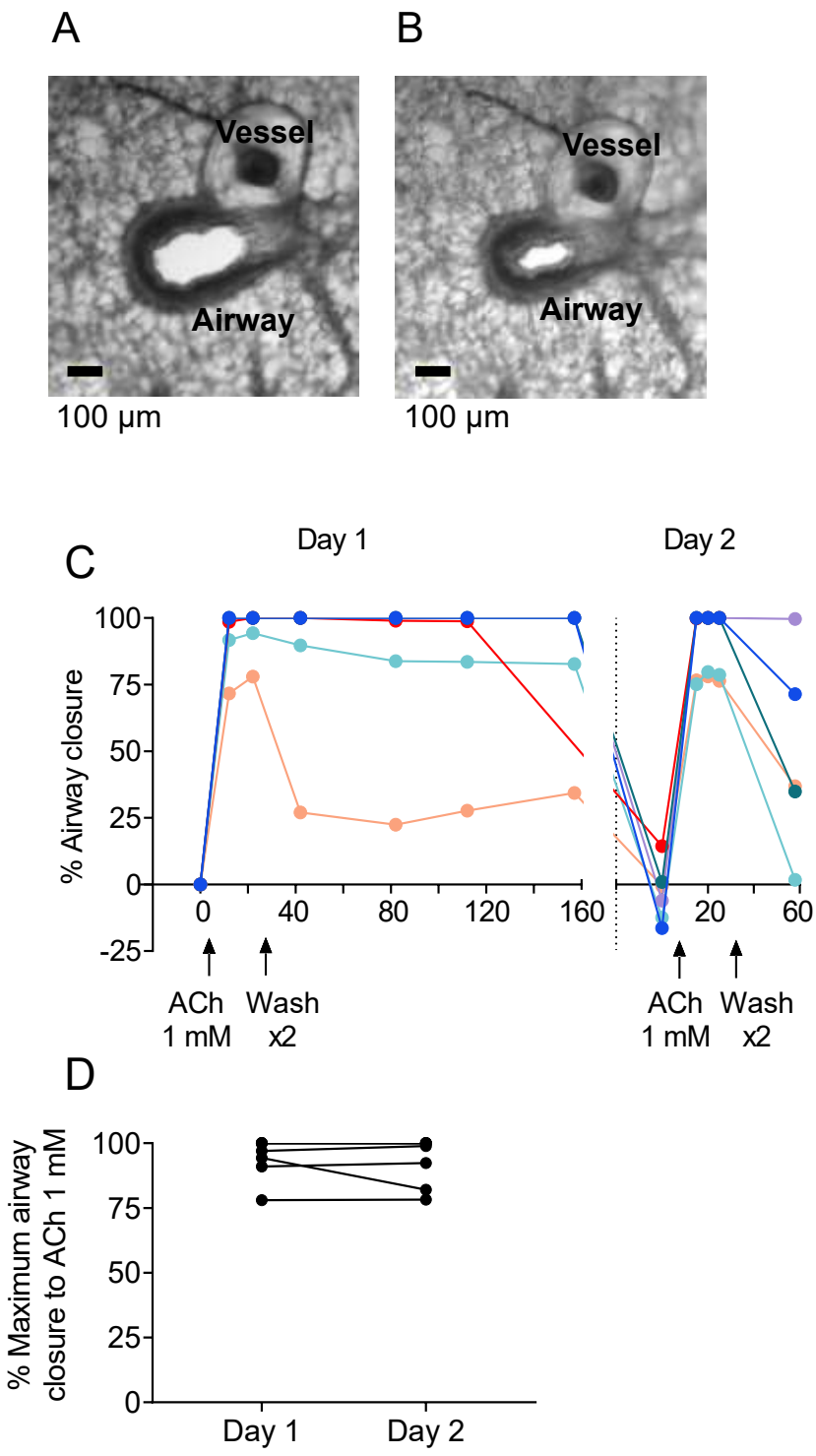


Figure 2-9 Comparison of ACh response between day 1 and day 2 post-isolation in PCLS

(A, B) Representative images of a small airway in a PCLS generated from a naïve rat at day 1 post-isolation **(A)** at baseline and **(B)** 10 min after ACh (1 mM) addition (airway diameter: 337 µm; 80% closure to ACh). **(C)** Representation of airway contraction over time and **(D)** quantification of the maximum contraction elicited by ACh (1 mM) of the same airways followed on day 1 and day 2 post-isolation, N=1 naïve rat, n=6 airways.

2.6.4.4 Day 2 post-isolation: PCLS experiments

On day 2 post-isolation, selected PCLS were washed twice and equilibrated in 1 ml of ECS at 37°C for 1 h in their 24-well plate as described in **2.6.4.2.** After 1 h, the medium volume was changed to 800 µl and the plate was taken under the light microscope maintained at 37°C. Airways selected on day 1 post-isolation were tracked down and baseline images taken. Compounds diluted in ECS were added to each well in a cumulative manner using a pipette, without washing between drug additions as the mechanical effect of washing could induce airway contraction. Snapshots were manually taken at specified timepoints to assess airway contraction. Specific protocols are detailed in individual result chapters. At the end of the experiment, slices were incubated with ACh (1 mM) for 10 min to evaluate airway viability and maximum contractility following drug treatment. All agonist/antagonist experiments were conducted according to the same procedure represented in (**Figure 2-10**).

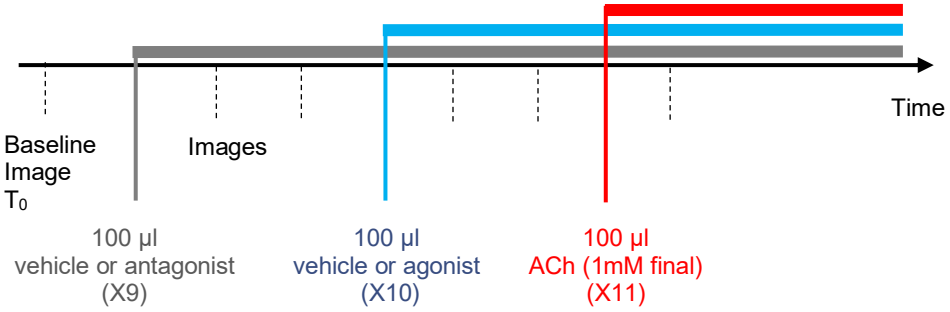


Figure 2-10 Protocol of small airways contraction experiments using PCLS

Dashed lines: images taken of each airways at appropriate timepoints; (X..): dilution of the drug solution added to each well.

2.6.4.5 Analysis of PCLS experiments

For each agonist/antagonist PCLS experiment, a single 24-well plate was imaged with up to 40 airways originating from one animal, with all conditions assessed at the same time with a minimum of 3 airways per condition. This was repeated with PCLS from different animals. Data were plotted as the percentage closure calculated from baseline T_0 over time. For analysis, the percentage closure ("% airway closure") elicited by an agonist was determined as the ratio between the lumen area right before adding the agonist (baseline T_1) and the lumen area at each timepoint after agonist addition. This was normalised to the final ACh response to account for inter-airway variability and the maximum contraction during the agonist incubation period was referred to as "% of ACh contraction". Airways from different animals were grouped for statistical analysis to account for inter-airway variability.

2.6.4.6 Validation of the PCLS technique

To validate the PCLS technique, a preliminary cumulative concentration-response to the stable muscarinic agonist carbachol (CCh) was conducted. Selected airways contracted in a concentration-dependent manner to CCh with a -logEC_{50} value of 6.2 (**Figure 2-11**) similar to published values (Brown et al. 2013; Gustafsson and Persson 1989) and therefore validating the technique.

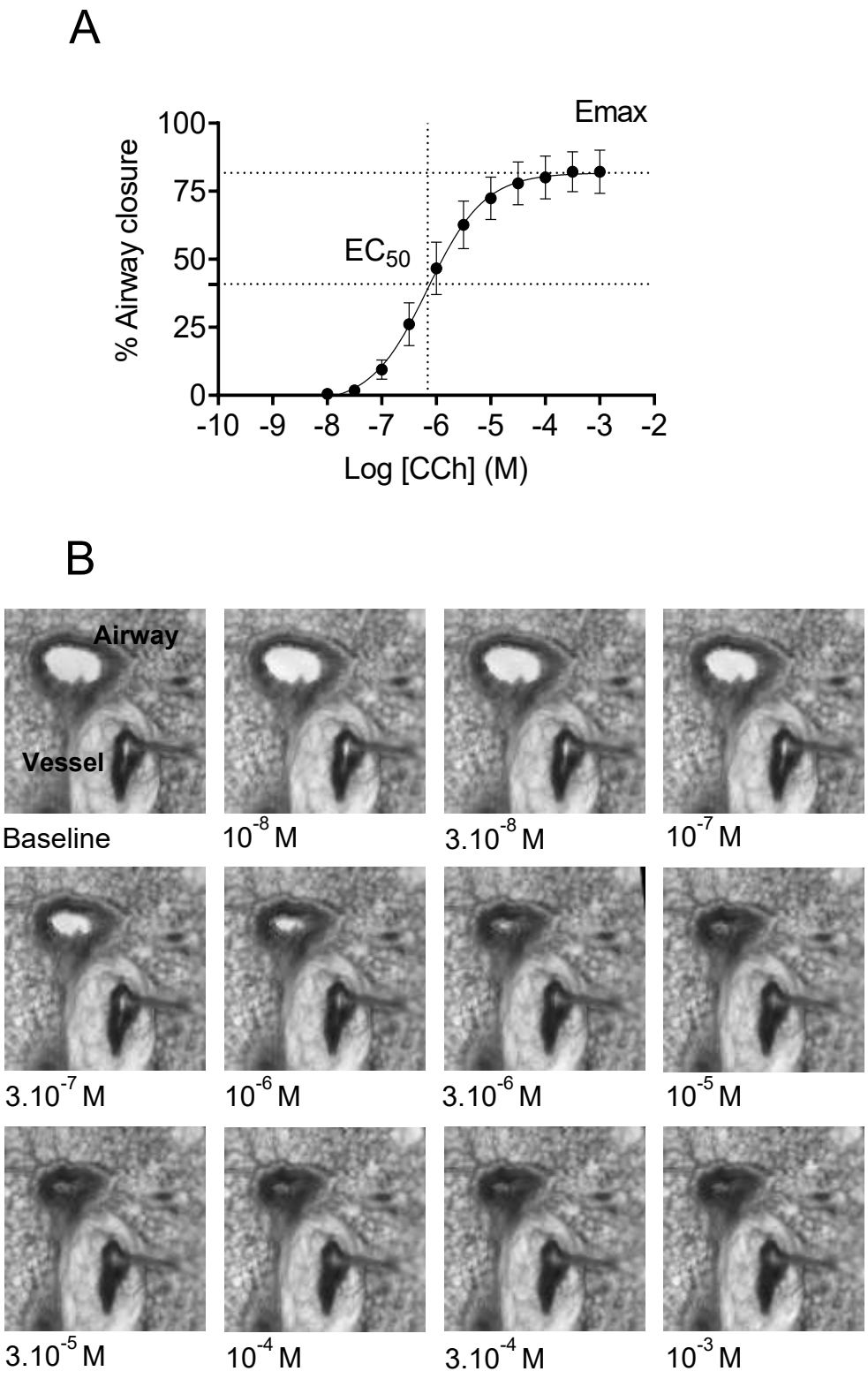


Figure 2-11 Validation of the PCLS technique: carbachol concentration-response

Rat PCLS were incubated on day 2 post-isolation with carbachol (CCh) in a cumulative manner and airway contraction monitored by light microscopy. **(A)** Concentration-response curve of the contraction elicited by CCh. Data expressed as mean ± S.E.M., N=1 rat, n=6 airways. **(B)** Representative images of an airway contracting to increasing concentrations of CCh.

2.7 Statistics

Statistical analysis was performed using Prism Software version 8.0 (GraphPad, USA). Statistical tests are specified in each individual result chapters.

2.8 Pharmacological compounds

The following table summarises the main pharmacological compounds used in this thesis.

	Compound	Potency/Selectivity	References
TRPV4 agonist	GSK1016790A	pEC50 7.7 – 8.7 (mouse, rat, human) Selectivity for TRPV4 tested in *Trpv4*$^{-/-}$ mouse tissues and against TRPM8, TRPA1 human channels	(Thorneloe et al. 2008; Willette et al. 2008; Alexander et al. 2013)
TRPV4 antagonist	GSK2193874	pIC_{50} 7.4 – 8.7 (human, mouse, rat) Selectivity tested against ~200 human proteins in cell lines and against TRPA1 and TRPV1 in guinea pig tissues	(Thorneloe et al. 2012; Cheung et al. 2017; Bonvini et al. 2016)
P2X3 agonist	αβ-MeATP	pEC_{50} ~ 6 Selective for P2X1 and P2X3	(Lewis et al. 1995; Cockayne et al. 2000)
P2X3 P2X2/3 antagonist	AF-353	pIC_{50} 7.3 – 8.5 (human, rat) Selectivity tested against human P2X1, P2X2, P2X4, P2X5, P2X7 in cell lines and against TRPA1 and TRPV1 in guinea pig tissues	(Gever et al. 2010; Bonvini et al. 2016)
Panx1 blocker	Probenecid	pIC_{50} ~ 3.8 (mouse)	(Silverman, Locovei, and Dahl 2008)
P2X4 antagonist	5-BDBD	pIC_{50} ~ 6.1 (rat)	(Coddou et al. 2019)
PAR2 agonist	2f-LIGRLO-NH2	pEC_{50} 6.5 – 7.0 (rat) Selectivity tested in *Par2*$^{-/-}$ mice and against human PAR1 and PAR4 in cell lines	(McGuire et al. 2004; Kawabata et al. 2004)
PAR2 antagonist	P2pal-18S	pIC_{50} ~ 6.8 (rat) Selectivity tested in *Par2*$^{-/-}$ mice and against PAR1 and PAR4 in cell lines	(Sevigny et al. 2011; Shearer et al. 2016)

Table 4 Pharmacological compounds

3 Investigating the effect of TRPV4 on rat airway sensory nerves

3.1 Rationale

In atopic asthmatics, allergen inhalation challenge can provoke a prolonged episode of airway narrowing named the late asthmatic response (LAR) (Diamant et al. 2013). The LAR has proven to be a relevant endpoint to study therapies for atopic asthma (Gauvreau, El-Gammal, and O'Byrne 2015) and yet its mechanism remains unclear. Because the LAR is associated with airway inflammation and reduced by corticosteroids, it is thought to result from the recruitment of inflammatory cells (Gauvreau, Watson, and O'Byrne 1999; Lommatzsch et al. 2006; Cockcroft and Murdock 1987; Kidney et al. 1997; Becky Kelly, Busse, and Jarjour 2000; Gauvreau et al. 1996; Inman et al. 2001; Leigh et al. 2002). However, it is unclear how inflammatory cells cause airflow limitation. One hypothesis is that recruited eosinophils and basophils release CysLTs in the airways (Gauvreau, El-Gammal, and O'Byrne 2015). However, the anti-IL-5 mepolizumab had no effect on the LAR, questioning the role of eosinophils (Leckie et al. 2000), and not all patients respond to LTRAs (Roquet et al. 1997; Hamilton et al. 1998; Diamant et al. 1999; Leigh et al. 2002; Davis et al. 2009).

Evidence suggests that airway nerves could be the link between inflammation and airflow limitation. The LAMA tiotropium was shown to be effective in improving symptoms and baseline lung function in asthmatic patients (Peters et al. 2010; Kerstjens et al. 2015; Paggiaro et al. 2016). Importantly, the Respiratory Pharmacology group has shown that tiotropium and anaesthesia abolished the LAR in a rat OVA model (Raemdonck et al. 2012), suggesting that the LAR is driven by a nerve reflex, whereby allergen challenge results in activation of airway sensory nerves and a cholinergic reflex bronchospasm. However, the mechanisms driving this reflex are currently unknown.

Transient receptor potential channel V4 (TRPV4) is a cation-permeable ion channel and a known activator of airway sensory nerves (Bonvini et al. 2016). TRPV4 activation seems to indirectly stimulate airway sensory neurons by inducing the release of ATP through Panx1 channels and the activation of P2X3-P2X2/3 receptors expressed on the neurons (Bonvini et al. 2016) (**Figure 3-1**). Interestingly, ATP levels have been found elevated in the airways of asthmatic patients after allergen challenge (Idzko et al. 2007). Furthermore, PAR2 activation has been shown to activate or sensitise TRPV4 and to elicit a sensory nerve signal (Sipe et al. 2008; Grant et al. 2007; Poole et al. 2013; Zhao et al. 2014; 2015; Grace et al. 2014) and PAR2 receptors as well as PAR2 agonists have been found elevated in the airways of

asthmatic patients (Sedgwick et al. 1991; S. E. Wenzel, Fowler, and Schwartz 1988; Aubier et al. 2016; Knight et al. 2001; Allard et al. 2014).

Since both TRPV4 upstream activators and downstream mediator are upregulated in asthma, it was hypothesised that TRPV4 could be a driver of the nerve reflex causing the LAR. This thesis aimed to investigate this hypothesis using an OVA model of the LAR established in Brown Norway rats. However, the effect of TRPV4 on airway sensory nerve activation has been previously characterised in guinea pigs, mice and human but not rats (Bonvini et al. 2016). Therefore, the first aim of this thesis was to confirm whether TRPV4 can activate airway sensory nerves in Brown Norway rats.

3.2 Hypothesis

TRPV4 activates airway sensory nerves in naïve rats via inducing the release of ATP through Panx1 channels acting on P2X3-P2X2/3 receptors (**Figure 3-1**).

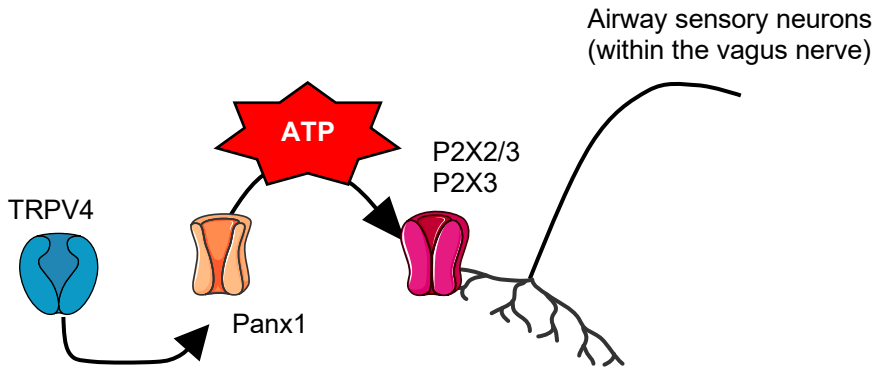


Figure 3-1: Hypothesis: TRPV4 activates airway sensory nerves via the ATP-P2X3 axis

3.3 Aims

- Investigate whether TRPV4 activation can stimulate airway sensory nerves in naive rats using electrophysiological techniques *in vitro* and *in vivo.*
- Investigate whether this effect is mediated via the release of ATP through Panx1 channels and the activation of P2X3-P2X2/3 receptors *in vitro*.

3.4 Methods

3.4.1 Effect of TRPV4 on isolated vagus depolarisation

The effect of TRPV4 activation was tested on the depolarisation of rat vagal nerves *in vitro*, using the isolated vagus preparation detailed in **2.2.1** generated from naïve rats.

3.4.1.1 Effect of TRPV4, Panx1 and P2X3 antagonists on TRPV4-mediated vagus depolarisation

Vagal nerves were isolated from naïve rats. Each segment was incubated with the TRPV4 agonist GSK1016790A (300 nM) for 2 min. This was repeated after a wash to ensure reproducibility of the response. The nerve was subsequently incubated with the antagonist for 10 min, either the TRPV4 antagonist GSK2193874 (10 μM), Panx1 blocker probenecid (1 mM), P2X3-P2X2/3 antagonist AF-353 (10 μM) or the vehicle (0.1% DMSO). The TRPV4 agonist was then reapplied for 2 min in presence of the antagonist. After washing, the nerve was re-incubated with the TRPV4 agonist to establish viability and recovery of the response.

Compound concentrations were chosen based on concentration-response experiments conducted in guinea pigs. For the TRPV4 agonist, this corresponded to the concentration eliciting submaximal depolarisation of the vagus nerve. For the TRPV4 and P2X3-P2X2/3 antagonists, this corresponded to the concentration eliciting maximum blockade of their respective agonist GSK1016790A or αβ-MeATP (Bonvini et al. 2016). The concentration of the Panx1 blocker probenecid was chosen according to the published report (Silverman, Locovei, and Dahl 2008).

Selectivity of the TRPV4 agonist and TRPV4, P2X3-P2X2/3 antagonists have been characterised in the literature (Thorneloe et al. 2012; Gever et al. 2010; Thorneloe et al. 2008). In-house studies confirmed their selectivity at the concentrations used against TRPA1 and TRPV1-induced vagus depolarisation in guinea pigs (Bonvini et al. 2016). Of note, the antagonist AF-353 selectively blocks P2X3 receptor units in P2X3 homotrimeric and P2X2/3 heterotrimeric receptors. The table presented in **2.8** summarises the properties of the compounds used in this thesis.

3.4.1.2 Effect of the ATP analogue αβ-MeATP on vagus depolarisation

Vagal nerves were isolated from naïve rats. Each segment was incubated with the stable ATP analogue αβ-MeATP (100 μM) for 2 min. This was repeated after a wash to ensure reproducibility of the response. The nerve was then incubated with the P2X3-P2X2/3 antagonist AF-353 (10 μM) for 10 min. The ATP analogue αβ-MeATP was subsequently

reapplied for 2 min in presence of the P2X3 antagonist. After washing, the nerve was re-incubated with αβ-MeATP to establish viability and recovery of the response.

The ATP analogue αβ-MeATP preferentially binds to P2X1 and P2X3 receptors (Lewis et al. 1995; Cockayne et al. 2000). The concentration of αβ-MeATP was chosen based on concentration-response experiments as the concentration eliciting submaximal vagus depolarisation in guinea pigs (Bonvini et al. 2016).

3.4.1.3 Validation of TRPV4 antagonist selectivity

The selectivity of the TRPV4 antagonist GSK2193874 was tested in the rat species against TRPV1-induced vagal depolarisation. The rat vagus nerve was incubated with the TRPV1 agonist capsaicin (1 μM) for 2 min. This was repeated after a wash to ensure reproducibility of the response. The nerve was then incubated with the TRPV4 antagonist GSK2193874 (10 μM) for 10 min. The TRPV1 agonist was subsequently reapplied for 2 min in presence of the TRPV4 antagonist. After washing, the nerve was re-incubated with the TRPV1 agonist to establish viability and recovery of the response.

3.4.1.4 Vagus data analysis

Vagus data were expressed as mean ± S.E.M. depolarisation (mV) and percentage inhibition calculated for each antagonist. Statistical analysis was performed on the depolarisation (mV) data. Parametric paired t-tests compared the depolarisation elicited by the agonist in absence and presence of the antagonist.

3.4.2 Effect of TRPV4 on isolated tracheal nerve firing

The effect of TRPV4 activation was tested on compound action potential firing of the tracheal terminals of the RLN *in vitro,* using the isolated tracheal nerve preparation detailed in **2.2.2** generated from a naive rat.

The RLN was isolated *en bloc* with the upper airways from a naïve rat. The tracheal lumen was perfused with the vehicle (0.1% DMSO) for 10 min. After a wash, the TRPV4 agonist GSK1016790A (300 nM) was applied for 10 min. This was repeated to ensure reproducibility of the response. The tracheal lumen was then perfused with the TRPV4 antagonist GSK2193874 (10 μM) for 20 min. The TRPV4 agonist was subsequently reapplied for 10 min in presence of the TRPV4 antagonist. After washing, the tracheal lumen was re-incubated with the TRPV4 agonist to establish viability and recovery of the response.

Compounds were used at concentrations previously validated in isolated vagus experiments as described in **3.5.1**.

Data were analysed as nerve mass activity (μV.sec), reflecting the compound action potential firing of the RLN nerve connected to the trachea. Statistical analysis was not performed as only N=1 experiment was conducted.

3.4.3 Effect of TRPV4 on airway sensory Aδ-fibre firing

The effect of TRPV4 activation was tested on the action potential firing of airway sensory Aδ-fibres *in vivo*, using the single fibre preparation detailed in **2.2.3** in an anaesthetised naïve rat.

A naïve rat was anaesthetised, paralysed and artificially ventilated. Both vagi were disconnected from their central end (bilateral cervical vagotomy) to avoid vagal reflexes. After identification of an airway sensory Aδ-fibre and establishing baseline activity, the vehicle (1% EtOH in saline) was aerosolised for 1 min into the rat airways via a tracheal cannula. After 10 min, the TRPV4 agonist GSK1016790A (100 ng/ml corresponding to 152 nM) was aerosolised for 1 min. Airway sensory Aδ-fibre activity was observed for at least 30 min after TRPV4 agonist stimulation.

The concentration of the TRPV4 agonist was determined in previous concentration-response studies as the concentration eliciting submaximal airway sensory Aδ-fibre firing in guinea pigs (Bonvini et al. 2016).

Data were analysed as the frequency of action potential firing events (impulses/sec) of the airway sensory Aδ-fibre. Statistical analysis was not performed as only N=1 experiment was conducted.

3.5 Results

The first aim was to test whether TRPV4 activation can stimulate airway sensory nerves in naive rats. This was investigated using three complementary electrophysiological techniques.

3.5.1 Effect of TRPV4 on isolated vagus depolarisation

The effect of the TRPV4 agonist GSK1016790A was tested on the depolarisation of vagal nerves isolated from naïve rats. The TRPV4 agonist GSK1016790A (300 nM) provoked the depolarisation of rat isolated vagal nerves. The TRPV4 antagonist GSK2193874 (10 μM) inhibited this depolarisation by 78% (0.28 ± 0.07 mV vs 0.07 ± 0.03 mV) while its vehicle had no effect (**Figure 3-2**).

3.5.2 Selectivity of the TRPV4 antagonist against TRPV1-mediated vagus depolarisation

The selectivity of the TRPV4 antagonist GSK2193874 (10 μM) was tested against the TRPV1 agonist capsaicin (1 μM) mediated depolarisation of rat isolated vagal nerves. The TRPV4 antagonist did not inhibit TRPV1-induced depolarisation (0.36 ± 0.13 mV vs 0.39 ± 0.12 mV) (**Figure 3-3**).

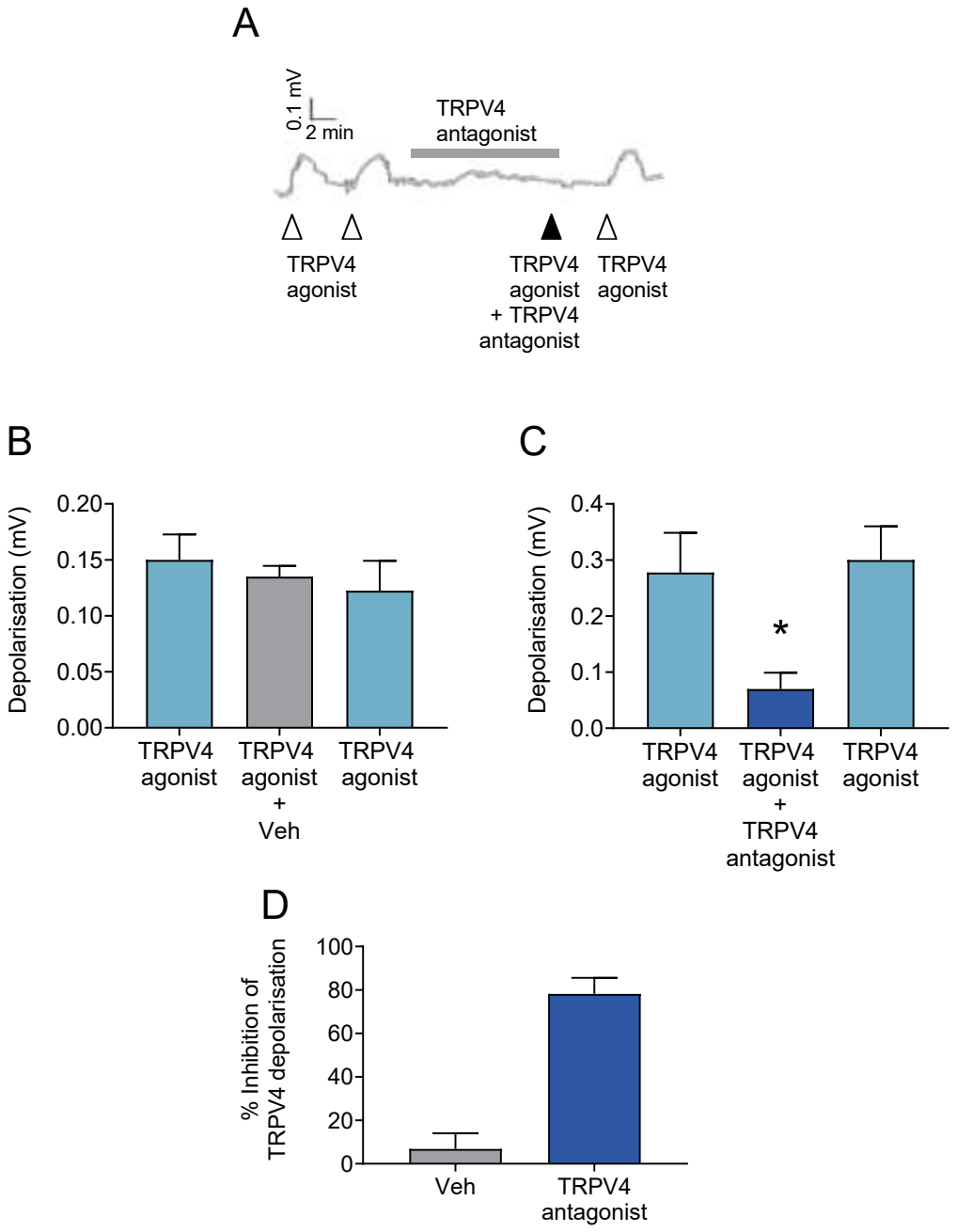


Figure 3-2: Effect of the TRPV4 agonist on isolated vagus depolarisation

Rat isolated vagal nerves were stimulated with the TRPV4 agonist GSK1016790A (300 nM) and the depolarisation of the nerve assessed in absence or presence of the TRPV4 antagonist GSK2193874 (10 µM) or its vehicle (0.1% DMSO). **(A)** Representative trace. **(B, C)** Quantification of the depolarisation (mV) induced by the TRPV4 agonist (left bar), in presence of the vehicle or TRPV4 antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). **(D)** Percentage inhibition of the TRPV4 agonist-induced depolarisation by the TRPV4 antagonist. Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *$p<0.05$ comparing the agonist response before and after antagonist treatment.

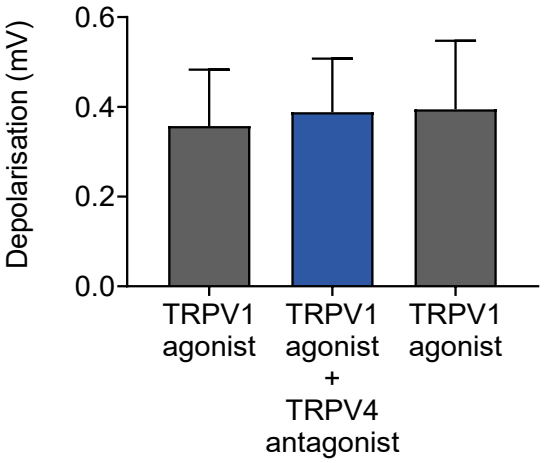


Figure 3-3: Effect of the TRPV4 antagonist against TRPV1-mediated vagus depolarisation

Rat isolated vagal nerves were stimulated with the TRPV1 agonist capsaicin (1 μM) and the depolarisation of the nerve assessed in absence or presence of the TRPV4 antagonist GSK2193874 (10 μM). Quantification of the mean depolarisation (mV) induced by the TRPV1 agonist (left bar), in presence of the TRPV4 antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *$p<0.05$ comparing the agonist response before and after antagonist treatment.

3.5.3 Effect of TRPV4 on isolated tracheal nerve firing

The ability of TRPV4 to specifically activate airway nerve terminals was interrogated using an isolated tracheal nerve preparation generated from a naïve rat. The TRPV4 agonist GSK1016790A (300 nM) increased compound action potential firing of the tracheal terminals of the recurrent laryngeal nerve (RLN) compared to the vehicle (0.1% DMSO). In the same preparation, the TRPV4 antagonist GSK2193874 (10 µM) inhibited this firing (**Figure 3-4**).

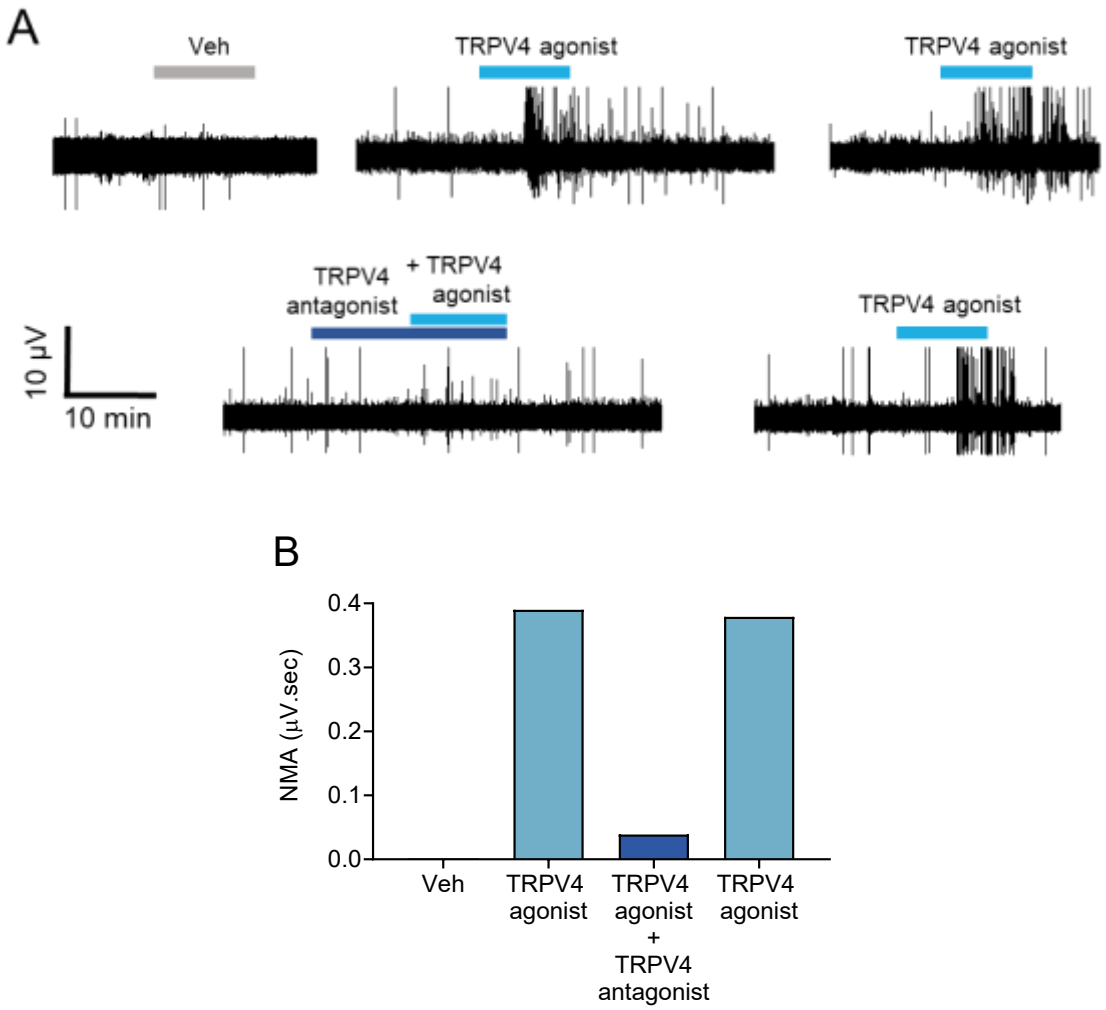


Figure 3-4: Effect of the TRPV4 agonist on isolated tracheal nerve firing

Upper airways (larynx, trachea, main bronchi) were isolated from a naïve rat and perfused with the TRPV4 agonist GSK1016790A (300 nM) or its vehicle (0.1% DMSO) and its effect recorded on compound action potential firing of the recurrent laryngeal nerve (RLN) attached to the trachea, in absence or presence of the TRPV4 antagonist GSK2193874 (10 µM). **(A)** Trace of RLN firing activity. **(B)** Quantification of nerve mass activity (µV.sec). Recovery of the agonist response was assessed at the end of the experiment. N=1 rat.

3.5.4 Effect of TRPV4 on airway sensory Aδ-fibre firing

The ability of TRPV4 to specifically activate airway sensory nerve terminals was subsequently tested *in vivo* using the single fibre preparation in an anaesthetised naïve rat. The interrogated fibre presented spontaneous activity in rhythm with the breathing pattern and responded to an aerosol of citric acid (0.3 M) and to lung hyperinflation/deflation. The fibre was therefore identified as an airway sensory Aδ-fibre (RAR). Nebulising the TRPV4 agonist GSK1016790A (100 ng/ml or 152 nM) directly into the rat trachea provoked an increase in the Aδ-fibre action potential firing frequency compared to the vehicle (1% EtOH) (**Figure 3-5**).

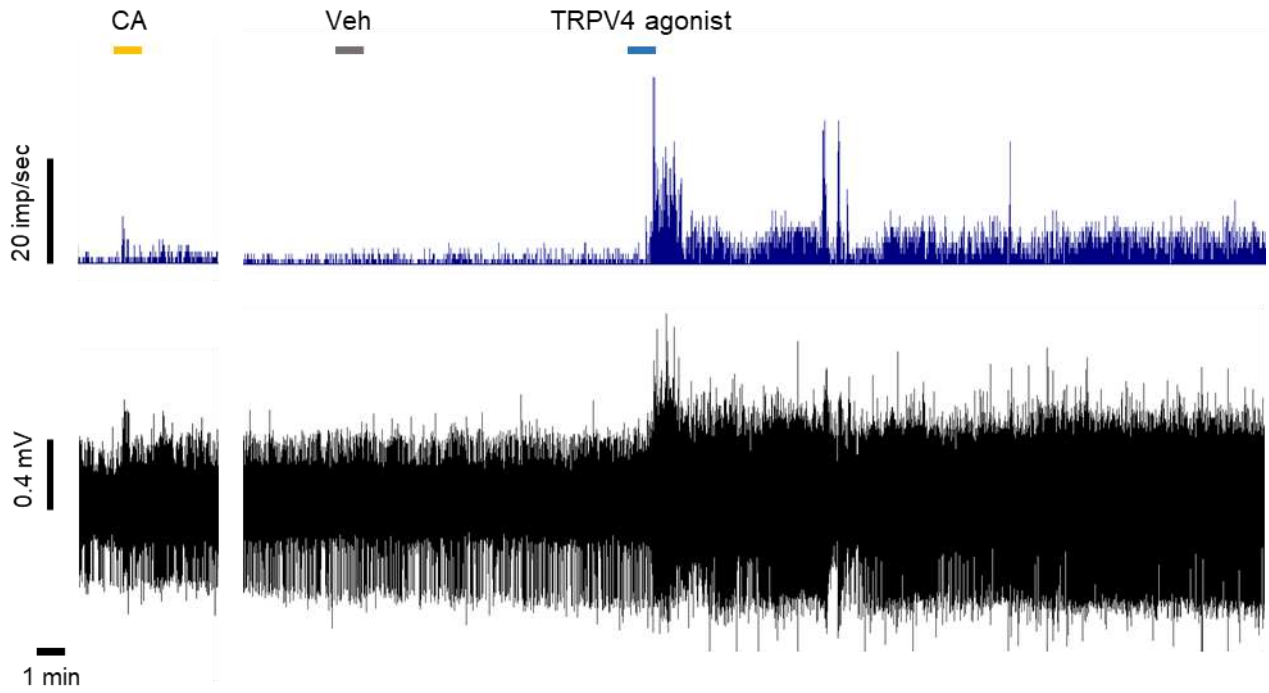


Figure 3-5: Effect of the TRPV4 agonist on airway sensory Aδ-fibre firing

A rat was anaesthetised, paralysed and both vagi cut at their central end. An airway sensory Aδ-fibre was identified and responded to citric acid (CA) (0.3 M). The vehicle (1% EtOH) was nebulised for 1 min and after 10 min, the TRPV4 agonist GSK1016790A (100 ng/ml or 152 nM) was nebulised for 1 min into the rat trachea and the effect assessed on Aδ-fibre action potential firing. The upper trace represents the frequency of action potential firing events above baseline activity (impulses/sec). The lower trace represents the raw recording of the nerve fibre potential (mV) throughout the experiment. N=1 rat.

3.5.5 Effect of αβ-MeATP on isolated vagus depolarisation

The second aim of this chapter was to investigate whether TRPV4 activates rat airway nerves via the release of ATP and activation of P2X3-P2X2/3 receptors.

The ability of ATP to depolarise rat vagal nerves via stimulating P2X3-P2X2/3 receptors was tested using the stable ATP analogue and P2X3 agonist αβ-MeATP on isolated vagal nerves generated from naïve rats. The P2X3 agonist αβ-MeATP (100 μM) provoked the depolarisation of rat isolated vagal nerves. The P2X3-P2X2/3 antagonist AF-353 (10 μM) inhibited this depolarisation by 48% (0.15 ± 0.02 mV vs 0.08 ± 0.01 mV) (**Figure 3-6**).

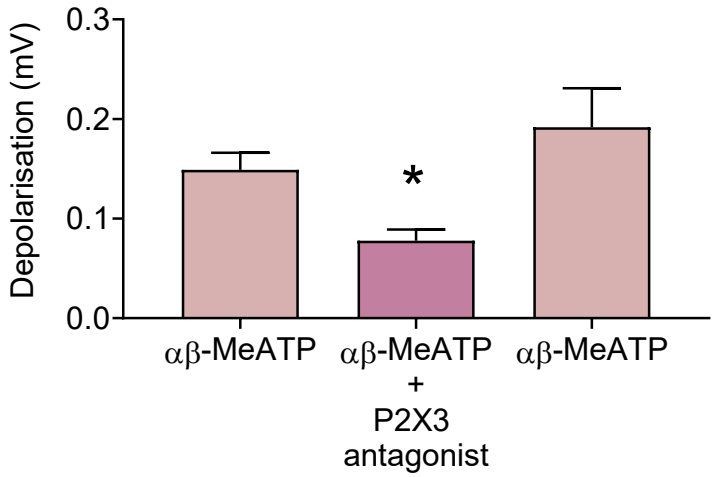


Figure 3-6: Effect of the P2X3 agonist αβ-MeATP on isolated vagus depolarisation

Rat isolated vagal nerves were stimulated with the P2X3 agonist αβ-MeATP (100 μM) and the depolarisation of the nerve assessed in absence or presence of the P2X3-P2X2/3 antagonist AF-353 (10 μM) or its vehicle (0.1% DMSO). Quantification of the depolarisation (mV) induced by the P2X3 agonist αβ-MeATP (left bar), in presence of the vehicle or P2X3-P2X2/3 antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *$p<0.05$ comparing the agonist response before and after antagonist treatment.

3.5.6 Effect of the Panx1 and P2X3 antagonists on TRPV4-induced vagus depolarisation

The effect of a Panx1 blocker and a P2X3-P2X2/3 antagonist was tested on TRPV4-induced rat vagus depolarisation. The Panx1 blocker probenecid (1 mM) inhibited TRPV4 agonist GSK1016790A (300 nM) induced depolarisation by 92% (0.19 ± 0.04 mV vs 0.01 ± 0.01 mV) while the vehicle (0.1% DMSO) had no effect (**Figure 3-7**). The P2X3-P2X2/3 antagonist AF-353 (10 µM) also inhibited TRPV4-induced depolarisation by 68% (0.17 ± 0.02 mV vs 0.05 ± 0.01 mV) (**Figure 3-8**). Percentage inhibitions are shown in (**Figure 3-9).**

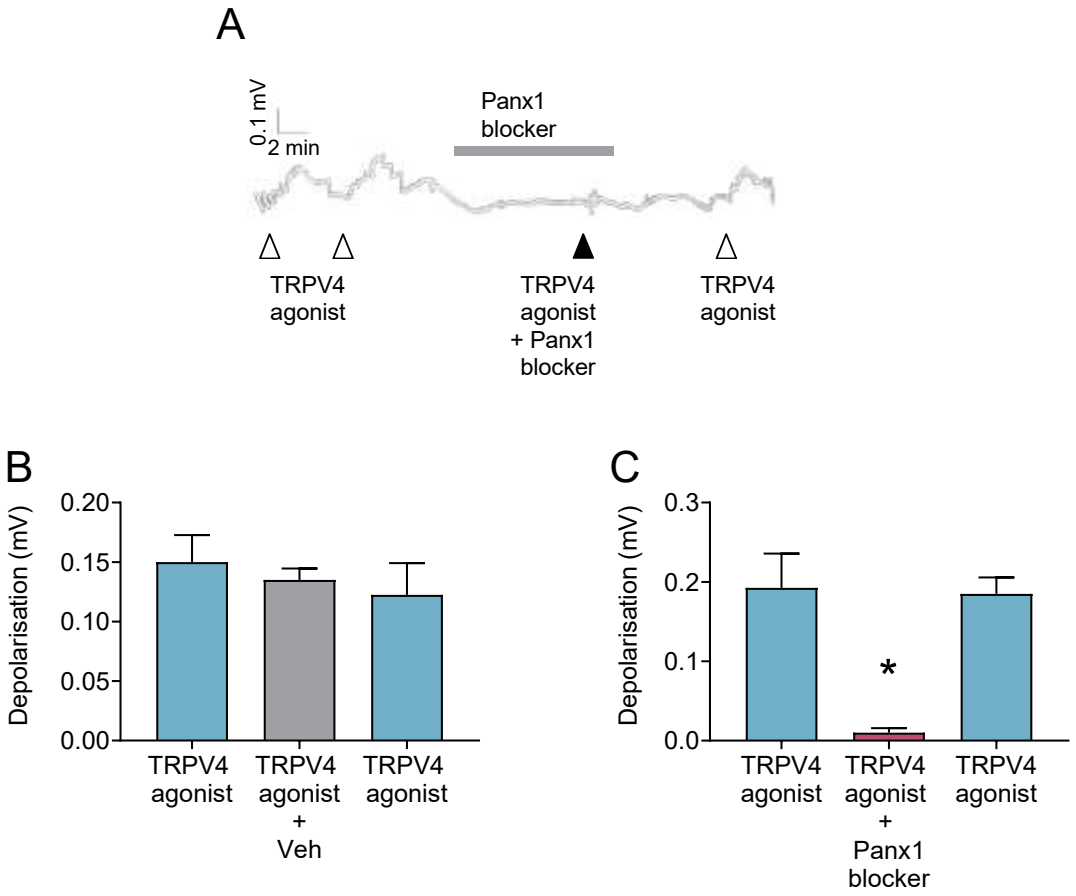


Figure 3-7: Effect of the Panx1 blocker on TRPV4-induced vagus depolarisation

Rat isolated vagal nerves were stimulated with the TRPV4 agonist GSK1016790A (300 nM) and the depolarisation of the nerve assessed in absence or presence of the Panx1 blocker probenecid (1 mM) or its vehicle (0.1% DMSO). **(A)** Representative trace. **(B, C)** Quantification of the depolarisation (mV) induced by the TRPV4 agonist (left bar), in presence of the vehicle or Panx1 blocker (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *p<0.05 comparing the agonist response before and after antagonist treatment.

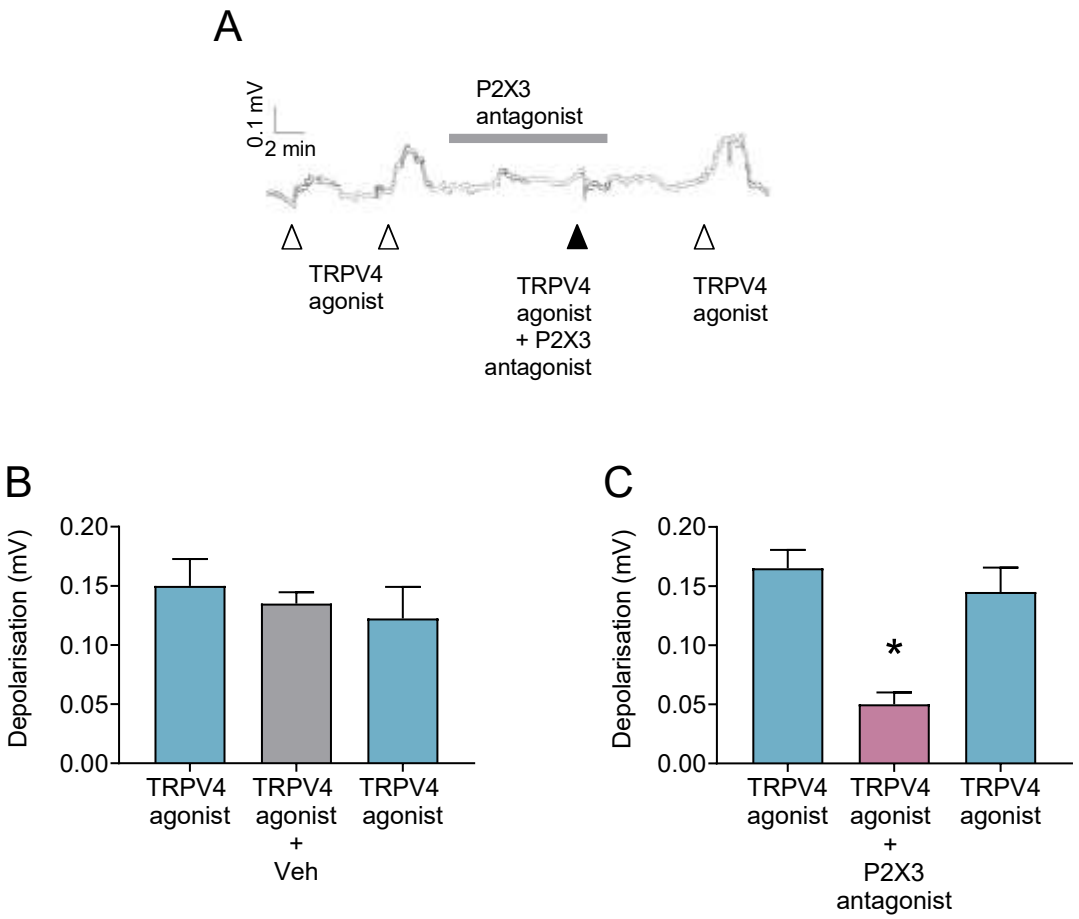


Figure 3-8: Effect of the P2X3 antagonist on TRPV4-induced vagus depolarisation

Rat isolated vagal nerves were stimulated with the TRPV4 agonist GSK1016790A (300 nM) and the depolarisation of the nerve assessed in absence or presence of the P2X3-P2X2/3 antagonist AF-353 (10 μM) or its vehicle (0.1% DMSO). **(A)** Representative trace. **(B, C)** Quantification of the depolarisation (mV) induced by the TRPV4 agonist (left bar), in presence of the vehicle or P2X3 antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *$p<0.05$ comparing the agonist response before and after antagonist treatment.

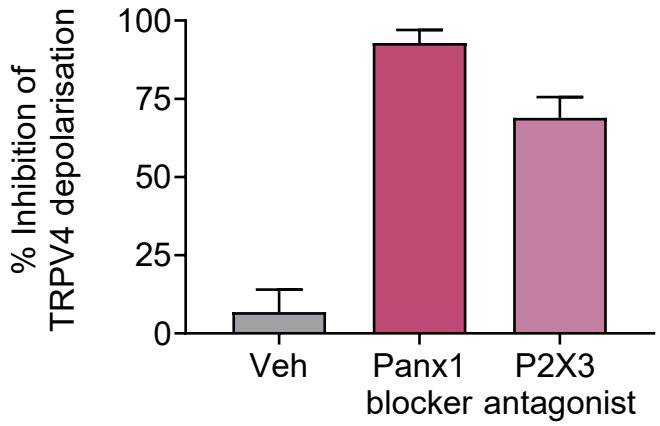


Figure 3-9: Inhibition of TRPV4-induced depolarisation by TRPV4 and P2X3 antagonists

Rat isolated vagal nerves were incubated with the TRPV4 agonist GSK1016790A (300 nM) in presence of the Panx1 blocker (1 mM) or P2X3-P2X2/3 antagonist AF-353 (10 μM) or their vehicle (0.1% DMSO). The graph represents the percentage inhibition of TRPV4 agonist-induced depolarisation for each antagonist. Data expressed as mean ± S.E.M., N=4 rats.

3.6 Discussion

This thesis investigated TRPV4 as a potential driver of the nerve reflex leading to the LAR, using an OVA-driven model of allergic airway disease in the Brown Norway rat. TRPV4 activation has been shown to activate airway sensory Aδ-fibres in guinea pigs and to depolarise mouse and human vagus nerves, but this has not been characterised in Brown Norway rats (Bonvini et al. 2016). The aim of this chapter was to test the effect of TRPV4 on the activation of airway sensory nerves in Brown Norway rats to determine if it is a suitable species/strain to study TRPV4 in the LAR.

The first part of this chapter tested the ability of TRPV4 to activate naïve rat airway sensory nerves using three complementary electrophysiology techniques. The selective synthetic agonist GSK1016790A was used to activate TRPV4 (Willette et al. 2008; Alexander et al. 2013; Thorneloe et al. 2008) as no specific endogenous agonist has been identified so far (White et al. 2016).

The TRPV4 agonist GSK1016790A elicited the depolarisation of rat isolated vagal nerves at a submaximal concentration determined from guinea pig studies (Bonvini et al. 2016). This effect was blocked by the TRPV4 antagonist GSK2193874. This antagonist is reported to be selective to TRPV4 in the literature (Thorneloe et al. 2012; Cheung et al. 2017) and previous in-house studies confirmed its selectivity against TRPA1 and TRPV1 channels in guinea pig tissues at the concentration used in this experiment (Bonvini et al. 2016). Because this antagonist would be taken forward in following *in vivo* studies, it was deemed necessary to further verify its selectivity in the rat species. Therefore, the selectivity of the antagonist was tested against the TRPV1 channel, TRPV4 closest homolog (Liedtke et al. 2000; Strotmann et al. 2000). The TRPV4 antagonist GSK2193874 did not inhibit the depolarisation induced by the TRPV1 agonist capsaicin in rat isolated vagal nerves, confirming its selectivity against TRPV1 channels in the rat. Overall, these results suggested that TRPV4 activation can depolarise naïve rat vagal nerves. These results translate to guinea pig, murine and more importantly to human tissues in the same preparation (Bonvini et al. 2016).

For the purpose of this thesis, it was important to ascertain whether TRPV4 could specifically activate the terminals of airway fibres comprised within the vagus nerve. While the vagus preparation is a useful tool to perform pharmacology studies and to translate results to human, it does not specifically assess the depolarisation of airway fibres as the vagus nerve comprises fibres originating from many different organs (Mazzone and Undem 2016). Receptors expression and signalling may also differ between axons and nerve terminals (Spaulding and Burgess 2017). In addition, nerve depolarisation may not reflect nerve activation, as a

compound can induce depolarisation at a subthreshold level insufficient to provoke action potential firing.

To circumvent these limits, the effect of the TRPV4 agonist was studied for the first time in an isolated tracheal nerve preparation generated from a naïve rat. The TRPV4 agonist GSK1016790A enhanced compound action potential firing of the tracheal terminals of the recurrent laryngeal nerve (RLN). This was blocked by the TRPV4 antagonist GSK2193874. Given that large airways record the highest density of innervation within the respiratory system (Jeffery and Reid 1973; A. Laitinen 1985; Mortola, Sant'ambrogio, and Clement 1975; Sant'Ambrogio et al. 1978) and that most of the trachea is innervated by the RLN (Ricco et al. 1996; Yamamoto et al. 1994; Canning et al. 2004; Liebermann-Meffert et al. 1999), these findings were particularly relevant in the context of airway reflexes. This preliminary result suggested that stimulation of TRPV4 channels can activate rat airway nerve terminals *in vitro*.

Both vagus and tracheal nerve preparations allow the study of nerve signals without the confounding factors of bronchoconstriction, mucus secretion, vascular effect or change in breathing that can indirectly stimulate airway nerves (Bergren 1997; Bonham et al. 1996; Kappagoda and Ravi 1989; Widdicombe 2003; Kollarik et al. 2003). This was an important factor to consider given that TRPV4 activation induces vasodilatation and increases vascular permeability (Willette et al. 2008). Isolated tracheal nerve activity can however be affected by tracheal muscle contraction (Weigand, Ford, and Undem 2012), but TRPV4 activation has not been shown to contract rat tracheas (McAlexander et al. 2014). Therefore, these *in vitro* data suggested that TRPV4 can directly activate airway nerve terminals in naïve rats.

Yet, these perfused techniques may not reflect the effect of TRPV4 activation *in vivo* and do not specifically assess sensory (afferent) signals. In this thesis, TRPV4 was hypothesised to stimulate airway sensory nerve fibres, thereby activating the afferent part of the LAR reflex. For this reason, the TRPV4 agonist was taken forward to the single fibre preparation using an anaesthetised rat to specifically assess airway sensory nerves activity. In this preparation, only fibres responding to chemical and mechanical stimuli delivered directly to the airways are interrogated and both vagi are disconnected from their central end, ensuring the recording of airway afferent signals. Airway sensory terminals are classified into two main categories, the Aδ- and C-fibres as described in **1.5.1.1**. Aδ-fibres are mostly mechanosensitive and C-fibres mostly chemosensitive, although subsets of chemosensitive Aδ-fibres and mechanosensitive C-fibres also exist (Mazzone and Undem 2016; Adcock et al. 2014). Mechanosensitive Aδ-fibres are mostly derived from the nodose ganglia and can take the form of RARs stretch receptors which respond to lung inflation and are very active during normal ventilation. RARs seem sensitive to any stimulus that exerts a mechanical pressure on the airways such as

bronchoconstriction, oedema or mucus secretion (Bergren 1997; Bergren and Sampson 1982; Canning et al. 2004; Sant'Ambrogio et al. 1978; Widdicombe 2003; Bonham et al. 1996; Kappagoda and Ravi 1989). In the guinea pig, TRPV4 activation only provoked the firing of interrogated RARs Aδ-fibres but not C-fibres (Bonvini et al. 2016) and therefore the effect of TRPV4 was only studied on rat Aδ-fibres in this thesis. Similar to guinea pigs, aerosolising a submaximal concentration of the TRPV4 agonist GSK1016790A into the airways of a naïve rat enhanced the action potential firing from a single airway sensory RAR Aδ-fibre compared to the vehicle. It should be noted that it was not possible to assess conduction velocity to conclusively identify that the interrogated fibre was an Aδ-fibre, however, the pattern of discharge and mechanosensitivity strongly suggested it. Furthermore, the fact that TRPV4 activates an RAR Aδ-fibre concurs with the activation of the RLN *in vitro*, as an important proportion of sensory fibres contained within the RLN originates from the nodose ganglia (Canning et al. 2004). Overall, this preliminary result suggested that TRPV4 can indeed activate the terminals of airway sensory nerves in naïve rats.

Another important limitation of this work is the number of repetitions carried out, with only N=1 isolated tracheal nerve and N=1 single fibre preparations. These techniques could only be performed by highly skilled electrophysiologists with limited availability during this thesis timeframe. These preparations are labour intensive and only one nerve fibre can be examined in a single animal. Furthermore, it was the first time that both preparations were set-up in the rat species, requiring important validation work. More importantly, the effect of TRPV4 has already been extensively characterised in the laboratory on guinea pig airway sensory Aδ- and C-fibres (Bonvini et al. 2016). Given that data obtained with rat isolated vagal nerves correlated with data obtained in guinea pigs, and the fact that previous studies demonstrated a good correlation between vagus depolarisation and airway sensory fibres firing (Nasra and Belvisi 2009), these complex techniques were only carried out for confirmation purposes in the rat.

The second aim of this chapter was to investigate whether TRPV4 activates rat airway nerves via the release of ATP through Panx1 channels, which then activates P2X3-P2X2/3 receptors as it was demonstrated in guinea pig, murine and human vagus nerve (Bonvini et al. 2016). This was investigated in the vagus preparation isolated from naïve rats using pharmacological tools at concentrations determined in previous studies in guinea pigs (Bonvini et al. 2016). The stable ATP analogue and P2X3 agonist αβ-MeATP depolarised the rat vagus nerve and this was reduced by the P2X3-P2X2/3 antagonist AF-353, confirming that ATP can stimulate rat vagal nerves by activating P2X3-P2X2/3 receptors. As expected, the TRPV4 agonist GSK1016790A-induced depolarisation was inhibited by the Panx1 channel blocker probenecid and by the selective P2X3-P2X2/3 antagonist AF-353 (Gever et al. 2010). This

suggested that TRPV4 activated rat vagal nerves via the release of ATP through Panx1 channels and the activation of P2X3-P2X2/3 receptors, similar to what was seen in human, guinea pig and mouse tissue (Bonvini et al. 2016).

As described in the introduction (**1.6.4.3** and **1.6.4.4**), it has been hypothesised that ATP, and by extent TRPV4, stimulates airway sensory neurons mostly via activating P2X2/3 heterotrimeric receptors, with P2X2/3 receptors being expressed on nodose-derived but not jugular-derived neurons (Kwong et al. 2008; Nassenstein et al. 2010; Bonvini et al. 2016). Accordingly, several TRPV4 agonists activated Aδ-fibres but not C-fibres in anaesthetised guinea pigs (Bonvini et al. 2016), concurring with the fact that Aδ-fibres are mostly derived from the nodose ganglia (Mazzone and Undem 2016) and that TRPV4 agonists induced calcium influx in nodose-derived but not jugular-derived guinea pig airway sensory neurons (Bonvini et al. 2016).

Multiple reports in many cell systems support the view that TRPV4 exerts its actions via the release of ATP as a secondary mediator. Studies have indeed measured ATP release upon TRPV4 activation by exogenous and endogenous stimuli in mouse, rat and human cells, in the airways, digestive tract and bladder (Rahman et al. 2018; Seminario-Vidal et al. 2011; Baxter et al. 2014; Bonvini et al. 2020; Mochizuki et al. 2009; Mihara et al. 2016; Shibasaki et al. 2014; Gevaert et al. 2007; Birder et al. 2007). Studies in human cells demonstrated that TRPV4-induced ATP release operates in a controlled manner via the opening of Panx1 channels (Rahman et al. 2018; Seminario-Vidal et al. 2011; Baxter et al. 2014). Although Panx1 blockers used in these studies are not selective, as for probenecid used in this thesis, these findings were confirmed in the literature using *Panx1*$^{-/-}$ mice (Seminario-Vidal et al. 2011; Bonvini et al. 2016). One of the cited mechanisms is that TRPV4 engages the intracellular RhoA kinase signalling pathway which phosphorylates Panx1 channels and thereby enhance their opening, leading to ATP release (Seminario-Vidal et al. 2011).

In vivo, the TRPV4 agonist GSK1016790A elicited an Aδ-fibre signal that was surprisingly prolonged. The fibre firing was sustained for at least 30 min in both anaesthetised guinea pigs (Bonvini et al. 2016) and in the anaesthetised rat shown in this chapter. The release of ATP could result in an amplifying and positive feedback mechanism, feeding a sustained nerve activation. Interestingly, studies have shown that TRPV4 can be sensitised to mechanical stimuli by extracellular ATP via the PLC-IP_3 pathway (Fernandes et al. 2008) and that ADP, a metabolite of ATP, can activate P2Y1 receptors and further open TRPV4 channels in glial cells (Rajasekhar et al. 2015). Sensitised TRPV4 channels could be opened by their cognate stimuli at lower thresholds and therefore elicit action potential firing upon stimuli that do not normally provoke a TRPV4-mediated signal, such as the mechanical forces exerted by breathing. This

prolonged and sustained firing makes TRPV4 a promising candidate for driving a prolonged nerve reflex in the LAR.

In contrast, one study found that TRPV4 stimulation did not elicit a current in airway sensory neurons isolated from rat vagal ganglia (Q. D. Gu et al. 2016). However, the same study showed that the TRPV4 agonist GSK1016790A induced rapid shallow breathing in anaesthetised rats and this was abolished by vagotomy, suggesting that TRPV4 activation can indeed trigger a nerve reflex. Several reasons might explain this discrepancy: 1) *In vitro* experiments were performed at 22^0C, temperature at which TRPV4 is not constitutively active (Güler et al. 2002; H. Watanabe, Vriens, et al. 2002); 2) An insufficient number of neurons may have been assessed, as the rat vagal ganglia is a fusion of both nodose and jugular ganglia and therefore nodose neurons expressing P2X2/3 heteromers and responding to TRPV4 stimulation would be diluted in the preparation. Supporting this hypothesis, the TRPV4 agonist only stimulated 17 out of 261 neurons tested in mouse sensory ganglia (Alexander et al. 2013); 3) The preparation may not have maintained non-neuronal cells surrounding the neurons, which likely express TRPV4 receptors and release ATP.

Overall, results from this chapter suggest that TRPV4 activation can stimulate airway sensory nerves in the naïve rat, via the release of ATP acting on P2X3-P2X2/3 receptors and translating results obtained with guinea pig, mouse and human vagus nerve. This validated the rat species as a relevant model to study the role of TRPV4 in driving a nerve reflex causing the LAR.

4 Effect of TRPV4 and P2X3 blockade on the LAR in a rat OVA model

4.1 Rationale

Previous studies from the group found that both anaesthesia and the anticholinergic tiotropium abolished the LAR in a rat OVA model of allergic airway disease (Raemdonck et al. 2012). This suggested that allergen challenge results in activation of airway sensory nerves and, after integration in the CNS, triggers an efferent cholinergic reflex causing the LAR. As tiotropium has recently been shown to improve baseline lung function and airflow limitation symptoms in asthmatic patients, this seems to be relevant to human (Peters et al. 2010; Kerstjens et al. 2015; Paggiaro et al. 2016). This thesis aimed to study potential mechanisms driving a nerve reflex leading to the LAR, using an OVA-driven model of allergic airway disease in the Brown Norway rat.

Studies conducted by the Respiratory Pharmacology group have highlighted that TRPV4 activates airway sensory nerves via the release of ATP activating P2X3-P2X2/3 receptors in guinea pigs, with translation in human vagus tissue (Bonvini et al. 2016). This led to hypothesise that TRPV4 could be a driver of the nerve reflex leading to the LAR.

Results from **Chapter 3** suggested that TRPV4 activation also stimulates airway sensory nerves in naïve Brown Norway rats, via the same mechanism of inducing ATP release and activating P2X3-P2X2/3 receptors. These results validated the possibility of using Brown Norway rats to investigate the role of the TRPV4-P2X3 nerve axis in the LAR.

4.2 Hypothesis

Allergen challenge results in the activation of the TRPV4-P2X3 axis which stimulates airway sensory nerves and provokes a cholinergic reflex bronchospasm and the LAR (**Figure 4-1**).

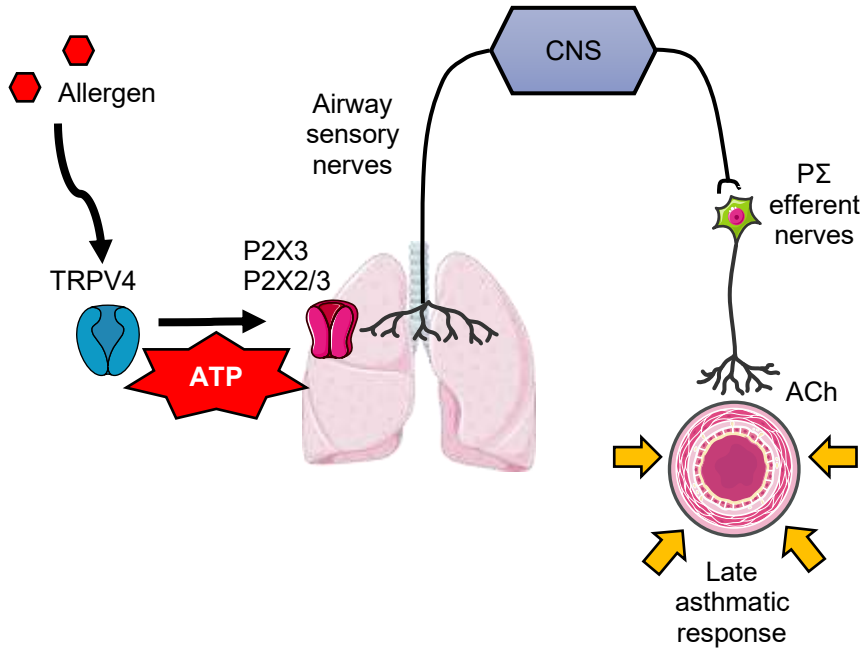


Figure 4-1 Hypothesis: the TRPV4-P2X3 axis drives a nerve reflex leading to the LAR
CNS: Central Nervous System. PΣ: Parasympathetic nerves.

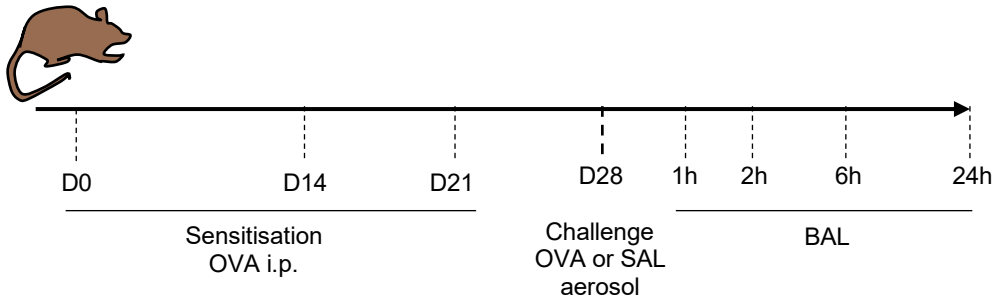
4.3 Aims

- Investigate the regulation of the TRPV4-P2X3 axis in the rat OVA model, by measuring ATP levels in BAL fluid at different timepoints after OVA challenge.
- Investigate whether the TRPV4-P2X3 axis is involved in the LAR, by testing TRPV4 and P2X3-P2X2/3 selective antagonists against the LAR in the rat OVA model.

4.4 Methods

4.4.1 Time-course of ATP release in the rat OVA model

ATP levels were measured in a time-course experiment in the rat OVA model. Male Brown Norway rats were sensitised on days D0, D14, D21 with OVA (in Alum™ diluted 1:1 in saline) as described in **2.3.1**. On D28, rats were challenged either with OVA or the vehicle SAL (saline) aerosolised for 30 min. After challenge, rats were euthanised with an overdose of pentobarbitone (200 mg/kg, i.p.) at 1, 2, 6 and 24 h post-challenge to harvest BAL samples as described in **2.3.3**, with N=6 rats/group per timepoint as shown in (**Figure 4-2**).



N	Sensitisation	Challenge	Timepoint of sample harvesting
6	OVA	SAL	1h
6	OVA	OVA	1h
6	OVA	SAL	2h
6	OVA	OVA	2h
6	OVA	SAL	6h
6	OVA	OVA	6h
6	OVA	SAL	24h
6	OVA	OVA	24h

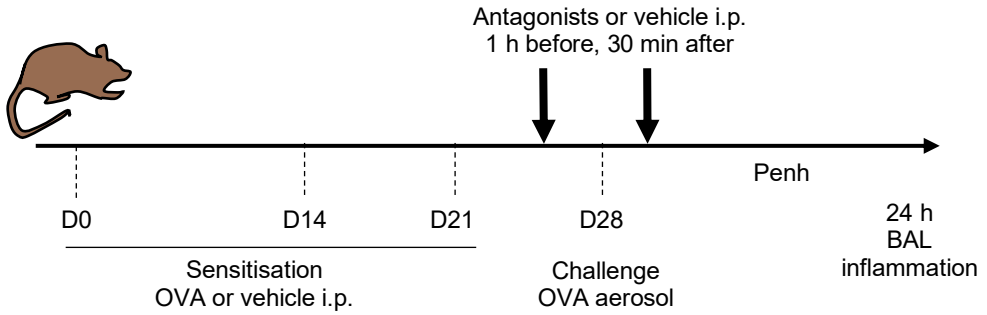
Figure 4-2 Protocol of the time-course experiment of the rat OVA model

ATP levels were measured in BAL samples using a luciferase/luciferin luminescent reaction according to the protocol described in **2.4.1**.

Data were expressed as raw luminescence (photons/sec) representing the levels of ATP in BAL samples. Non-parametric Mann-Whitney tests compared luminescence levels between SAL and OVA challenge for each timepoint.

4.4.2 TRPV4 and P2X3 antagonists in the rat OVA model

The TRPV4 and P2X3-P2X2/3 antagonists were tested against the LAR in the rat OVA model. Male Brown Norway rats were sensitised on days D0, D14, D21 with OVA or the vehicle SAL (Alum^{TM} diluted 1:1 in saline). On D28, all rats were challenged with OVA aerosol for 30 min as described in **2.3.1**. One hour before and 30 min after challenge, rats were dosed with either the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or the vehicle (6% HP-β-cyclodextrin in saline, i.p.), or the P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) or the vehicle (10% PEG400 in saline, i.p.), with N=4-8 rats/group (**Figure 4-3**). One hour after challenge, rats were placed in FWBP chambers to assess the LAR response by recording Penh from 1 to 6 h post-challenge as described in **2.3.2**. BAL cells were harvested 24 h post-challenge to perform inflammatory cells differential counts as described in **2.3.3**.



N	Sensitisation	Challenge	Treatment i.p.
4	SAL	OVA	Veh 6% HP-β-cyclodextrin
8	OVA	OVA	Veh 6% HP-β-cyclodextrin
8	OVA	OVA	TRPV4 antagonist GSK2193874
4	SAL	OVA	Veh 10% PEG400
8	OVA	OVA	Veh 10% PEG400
8	OVA	OVA	P2X3 antagonist AF-353

Figure 4-3 Protocol of TRPV4 and P2X3 antagonists study in the rat OVA model

4.4.2.1 Selection of antagonists dosing regimen

The doses of TRPV4 and P2X3-P2X2/3 antagonists were chosen based on previous dose-response experiments conducted against TRPV4 agonist GSK1016790A-induced cough in guinea pigs. At these doses, neither the TRPV4 nor P2X3-P2X2/3 antagonist inhibited TRPV1 agonist capsaicin-induced cough in guinea pigs (Bonvini et al. 2016). In the rat, GSK2193874 exhibited a half-life of 10 h and AF-353 a half-life of 1.63 h via oral gavage (Cheung et al. 2017; Gever et al. 2010). Compounds were dosed 1 h before and 30 min after challenge to

ensure sufficient exposure throughout the duration of the LAR and inflammatory cell recruitment.

4.4.2.2 Data analysis

To analyse the LAR, average Penh was calculated every 10 min and plotted over time. Statistical analyses were performed on Penh AUCs data. Non-parametric Mann-Whitney tests compared data between specified groups.

To analyse airway inflammation, statistical analyses were performed on BAL inflammatory cell counts expressed as (10^3 cells/ml). Non-parametric Mann-Whitney tests compared data between specified groups.

4.5 Results

4.5.1 Time-course of ATP release in the rat OVA model

The regulation of the TRPV4-P2X3 axis was assessed in the rat OVA model, by performing a time-course experiment of the rat OVA model without any antagonist treatment.

The release of TRPV4 downstream mediator, extracellular ATP, was assessed by a luciferin/luciferase assay in the BAL of OVA-sensitised rats after OVA or SAL challenge. ATP levels were found significantly elevated in the BAL of OVA-challenged compared to SAL-challenged rats at 6 h post-challenge (**Figure 4-4**).

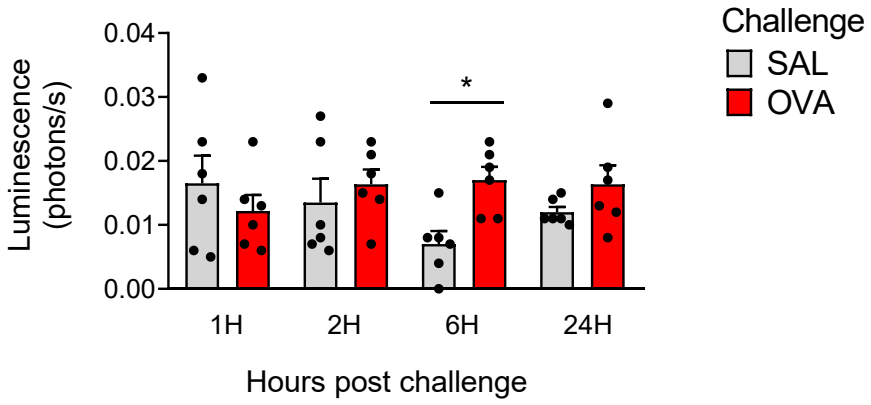


Figure 4-4 ATP levels in the airways in the rat OVA model

Rats were sensitised to OVA and challenged with OVA or SAL aerosol. BAL fluids were harvested at 1, 2, 6 and 24 h post-challenge to assess ATP levels by a luciferin/luciferase assay. Data expressed as mean ± S.E.M., N=6 rats per group per timepoint. Mann-Whitney test *p<0.05 comparing ATP levels between SAL and OVA-challenged rats for the same timepoint.

4.5.2 Effect of the TRPV4 antagonist on the LAR in the rat OVA model

The TRPV4 antagonist GSK2193874 was tested against the LAR in the rat OVA model. OVA challenge increased Penh AUC in OVA-sensitised compared to SAL-sensitised rats (111 ± 15 vs 2960 ± 586 Penh AUC), peaking at 3 h and lasting over 5 h post-challenge. The TRPV4 antagonist (300 mg/kg, i.p.) dosed 1 h before and 30 min post-challenge prevented this increase in Penh AUC by 72% compared to the vehicle (2960 ± 586 vs 826 ± 200 Penh AUC) (**Figure 4-5**).

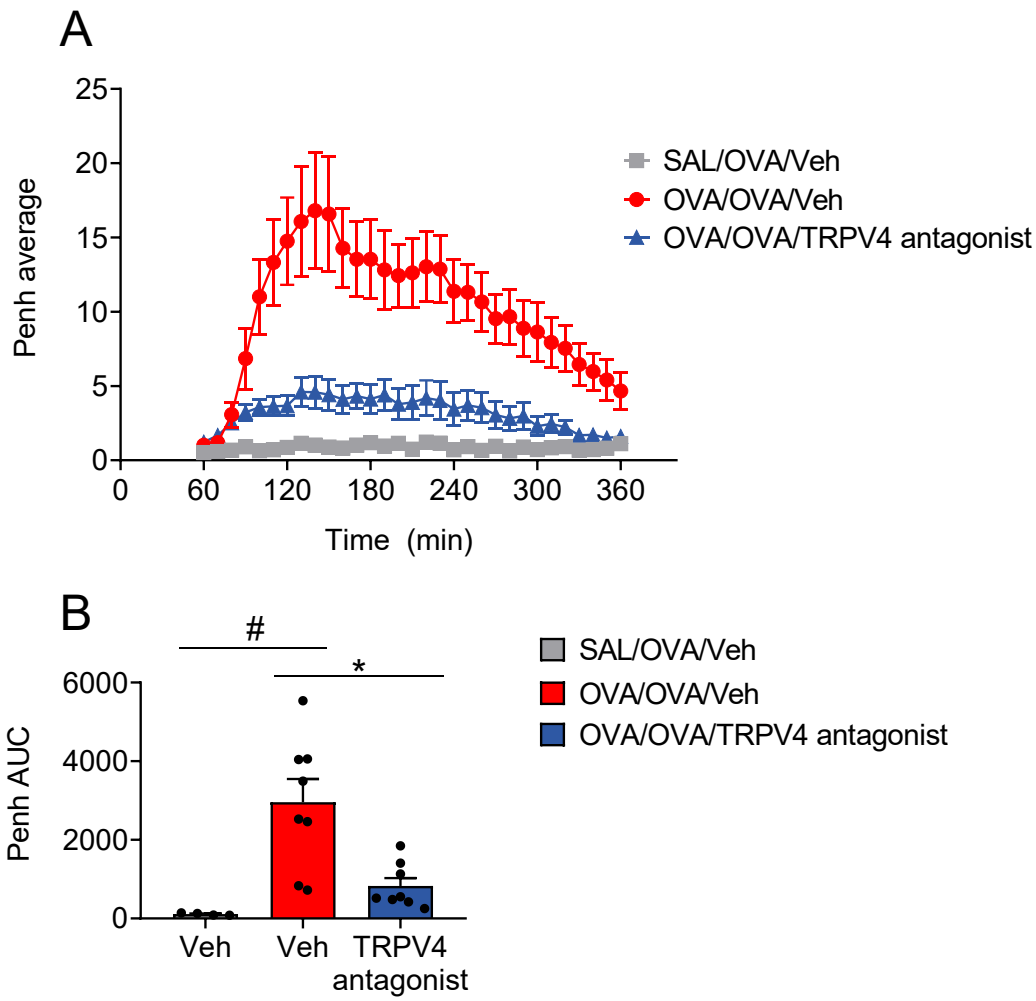


Figure 4-5 Effect of the TRPV4 antagonist on the LAR in the rat OVA model

Rats were sensitised with SAL or OVA and challenged with OVA aerosol. Rats were dosed with the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or the vehicle (6% HP-β-cyclodextrin, i.p.) 1 h before and 30 min after challenge. Penh was assessed 1 to 6 h post-challenge via FWBP. **(A)** Penh average over time. **(B)** Quantification of Penh AUC over the 5 h-recording period. Data expressed as mean ± S.E.M., N=4 rats in SAL/OVA and N=8 in OVA/OVA groups. Mann-Whitney test $^{\#}p<0.05$ SAL/OVA/Veh vs OVA/OVA/Veh and *p<0.05 antagonist vs vehicle response in OVA-sensitised rats.

4.5.3 Effect of the P2X3 antagonist on the LAR in the rat OVA model

In the same conditions, the P2X3-P2X2/3 antagonist AF-353 was tested against the LAR in the rat OVA model. The P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) dosed 1 h before and 30 min post-challenge prevented the increase in Penh AUC in OVA-sensitised rats by 85% compared to its vehicle (2947 ± 451 vs 452 ± 43 Penh AUC) (**Figure 4-6**).

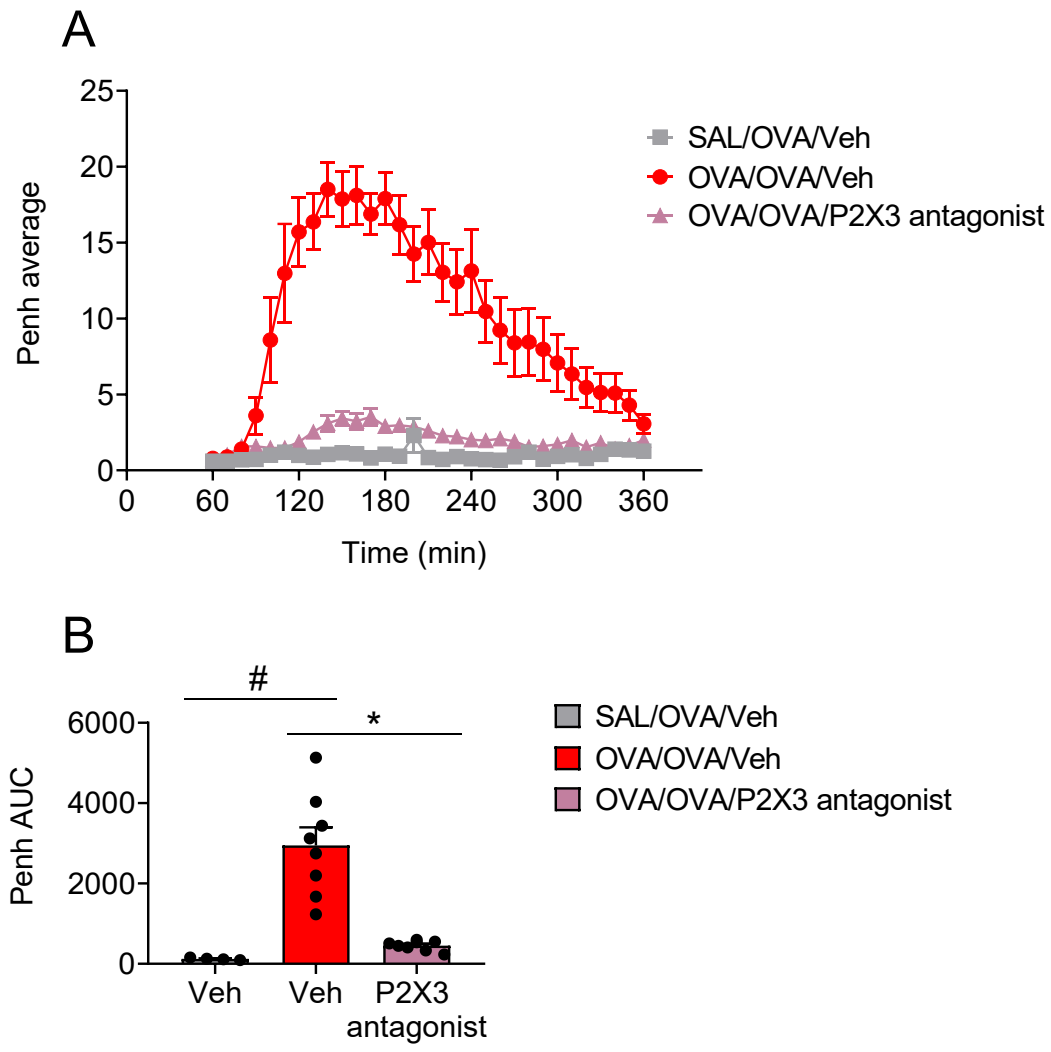


Figure 4-6 Effect of the P2X3 antagonist on the LAR in the rat OVA model

Rats were sensitised with SAL or OVA and challenged with OVA aerosol. Rats were dosed with the P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) or its vehicle (10% PEG400, i.p.) 1 h before and 30 min after challenge. Penh was assessed 1 to 6 h post-challenge via FWBP. **(A)** Penh average over time. **(B)** Quantification of Penh AUC over the 5 h-recording period. Data expressed as mean ± S.E.M., N=4 rats in SAL/OVA and N=8 in OVA/OVA groups. Mann-Whitney test $^{\#}p<0.05$ SAL/OVA/Veh vs OVA/OVA/Veh and *p<0.05 antagonist vs vehicle response in OVA-sensitised rats.

4.5.4 Effect of the TRPV4 antagonist on airway inflammation in the rat OVA model

OVA challenge provoked airway inflammation in OVA-sensitised compared to SAL-sensitised rats, with an increase in total leukocytes (40 ± 7 vs 287 ± 69 .10^3 cells/ml), eosinophils (1 ± 1 vs 45 ± 14 .10^3 cells/ml) and neutrophils (10 ± 4 vs 213 ± 52 .10^3 cells/ml) numbers in BAL samples at 24 h post-challenge. The TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) significantly reduced total leukocytes (287 ± 69 vs 58 ± 4 .10^3 cells/ml), eosinophils (45 ± 14 vs 3 ± 1 .10^3 cells/ml) and neutrophils (213 ± 52 vs 28 ± 3 .10^3 cells/ml) numbers compared to the vehicle (**Figure 4-7**).

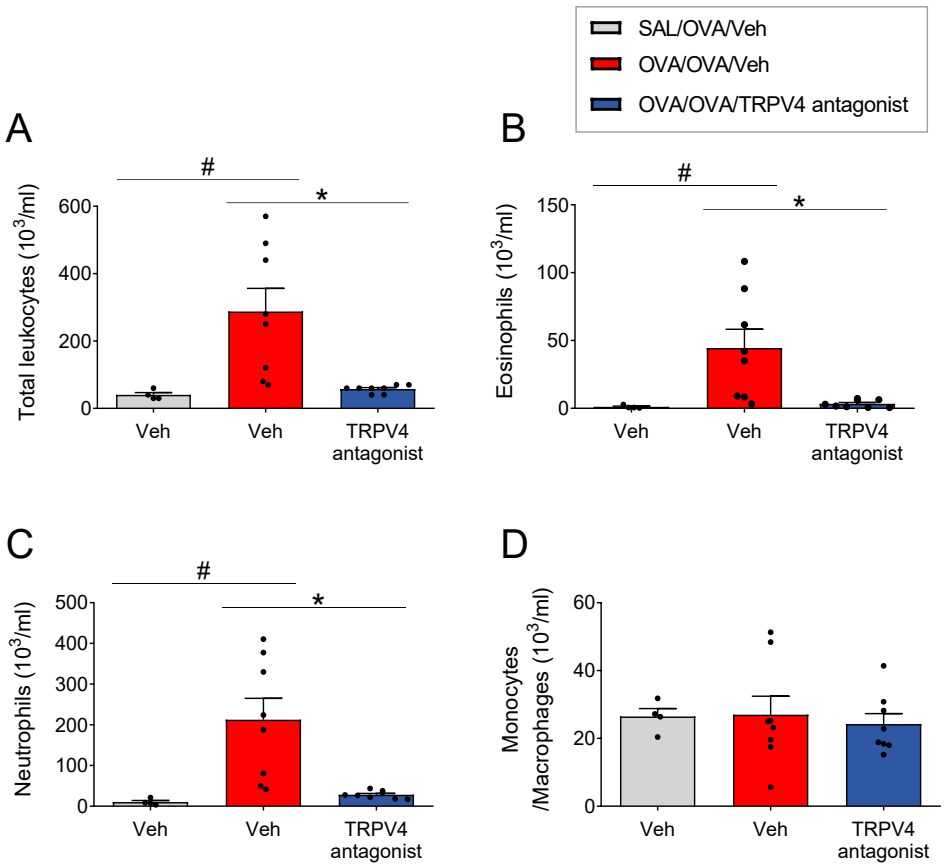


Figure 4-7 Effect of the TRPV4 antagonist on airway inflammation in the rat OVA model

Rats were sensitised with SAL or OVA and challenged with OVA aerosol. Rats were dosed with the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or its vehicle (6% HP-β-cyclodextrin, i.p.) 1 h before and 30 min after challenge. Airway inflammation was assessed in BAL at 24 h post-challenge by differential cell count: **(A)** Total leukocytes, **(B)** Eosinophils, **(C)** Neutrophils, **(D)** Monocytes/Macrophages. Data expressed as mean ± S.E.M., N=4 rats in SAL/OVA and N=8 in OVA/OVA groups. Mann-Whitney test $^{\#}p<0.05$ SAL/OVA/Veh vs OVA/OVA/Veh and $^{*}p<0.05$ antagonist vs vehicle response in OVA-sensitised rats. Legend in box above the graphs.

4.5.5 Effect of the P2X3 antagonist on airway inflammation in the rat OVA model

Similarly, the P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) significantly reduced total leukocytes (221 ± 38 vs 91 ± 7 .10^3 cells/ml), eosinophils (21 ± 5 vs 5 ± 1 .10^3 cells/ml) and neutrophils (174 ± 32 vs 61 ± 7 .10^3 cells/ml) numbers in BAL of OVA-sensitised rats compared to the vehicle at 24 h post OVA challenge (**Figure 4-8**).

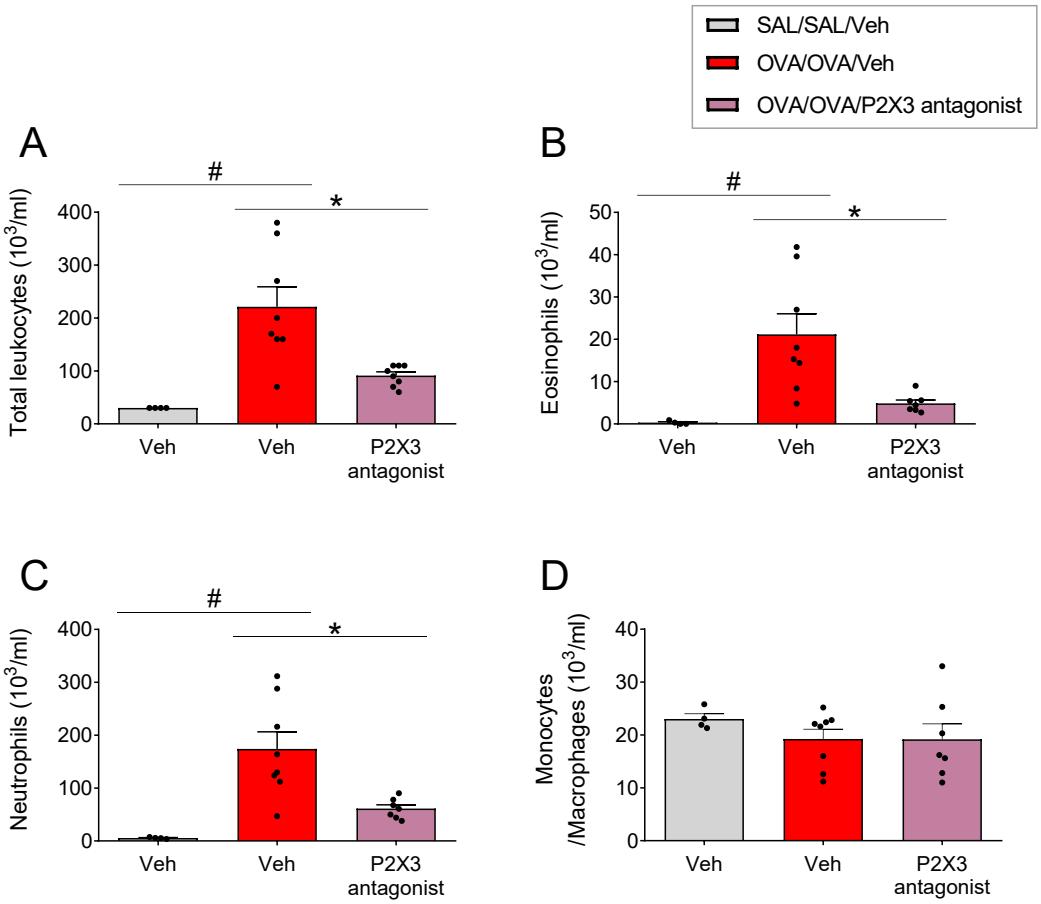


Figure 4-8 Effect of the P2X3 antagonist on airway inflammation in the rat OVA model

Rats were sensitised with SAL or OVA and challenged with OVA aerosol. Rats were dosed with the P2X3-P2X2/3 antagonist (30 mg/kg, i.p.) or its vehicle (10% PEG400, i.p.) 1 h before and 30 min after challenge. Airway inflammation was assessed in BAL at 24 h post-challenge by differential cell count: **(A)** Total leukocytes, **(B)** Eosinophils, **(C)** Neutrophils, **(D)** Monocytes/Macrophages. Data expressed as mean ± S.E.M., N=4 rats in SAL/OVA and N=8 in OVA/OVA groups. Mann-Whitney test $^{\#}p<0.05$ SAL/OVA/Veh vs OVA/OVA/Veh and *p<0.05 antagonist vs vehicle response in OVA-sensitised rats. Legend in box above the graphs.

4.6 Discussion

In asthmatic patients, the LAR is thought to be provoked by the recruitment of inflammatory cells as it is blocked by corticosteroids (Cockcroft and Murdock 1987; Kidney et al. 1997; Becky Kelly, Busse, and Jarjour 2000; Gauvreau et al. 1996; Inman et al. 2001; Leigh et al. 2002), yet the link between recruited cells and the prolonged bronchospasm is not clear. One hypothesis is that recruited eosinophils and basophils release CysLTs causing bronchoconstriction, oedema and mucus production (Gauvreau, El-Gammal, and O'Byrne 2015). However, reduction of eosinophils with the anti-IL-5 mepolizumab failed to inhibit the LAR and not all patients respond to LTRAs, therefore it is important to decipher alternative mechanisms responsible for prolonged bronchospasms seen in atopic asthma (Roquet et al. 1997; Hamilton et al. 1998; Diamant et al. 1999; Leigh et al. 2002; Davis et al. 2009; Leckie et al. 2000). The aim of this chapter was to investigate whether the TRPV4-P2X3 nerve axis could be one of the drivers of the LAR.

For this purpose, a well-characterised rat OVA model was used (Raemdonck et al. 2012; S. L. Underwood et al. 2002). In the literature, both rat and guinea pig species have been shown to exhibit early and late allergic responses to OVA, with inflammatory cell recruitment and pharmacological responses similar to that described in human allergen challenge studies (Zosky and Sly 2007; S. L. Underwood et al. 2002; S. Underwood et al. 1995; Smit et al. 2014; Stevenson and Belvisi 2008). While the LAR observed in guinea pigs does not seem reproducible between laboratories, it has been consistently modelled in rats in several groups (Stevenson and Belvisi 2008). In the Respiratory Pharmacology group, OVA inhalation provokes an immediate bronchospasm resolving within 30 min (EAR) and a delayed bronchospasm from 1 to 6 h post-challenge (LAR) in OVA-sensitised Brown Norway rats, associated with an influx of eosinophils and neutrophils within the airways (S. L. Underwood et al. 2002). Both immediate and late bronchospasms are responsive to gold standard asthma therapies, with the EAR being blocked by mast cell mediators antagonists and the LAR by ICS-LABA, emphasizing the relevance of this model (Raemdonck et al. 2012).

In addition, it was the observation that anaesthesia and tiotropium blocked the LAR in this rat model that led to hypothesise that a cholinergic reflex bronchospasm could be driving the LAR (Raemdonck et al. 2012). This was confirmed by another group who showed inhibition of the LAR by tiotropium in a guinea pig OVA model (Smit et al. 2014). The structurally distinct LAMA glycopyrrolate also reduced the LAR in the rat OVA model (unpublished data), supporting the engagement of muscarinic receptors rather than a possible off-target effect of tiotropium (Birrell et al. 2014). These findings and the observation that allergen challenge can stimulate airway sensory nerves in animal models including rats (Undem, Hubbard, and Weinreich 1993;

Riccio, Myers, and Undem 1996; G. Zhang et al. 2008; Potenzieri, Meeker, and Undem 2012) suggested that a cholinergic reflex could play an important role in the LAR and this could be studied in the rat OVA model. Another reason for choosing this model was that rat airway smooth muscle does not contract to CysLTs compared to guinea pigs (Canning and Chou 2008), with the LAR being attenuated by CysLTs blockade in guinea pigs but not in rats (Ishimura et al. 2008; Raemdonck et al. 2012). Concurrently, TRPV4 activation has been reported to contract large airways via the release of CysLTs in human and guinea pigs, but not in rats (McAlexander et al. 2014). The rat species was therefore advantageous to study nerve reflexes induced by TRPV4 without the confounding factor of CysLTs provoking local bronchospasm, and at the same time this enabled to study another endotype leading to the LAR than involving CysLTs (Roquet et al. 1997; Hamilton et al. 1998; Diamant et al. 1999; Leigh et al. 2002; Davis et al. 2009).

Prior to testing the effect of TRPV4 and P2X3-P2X2/3 antagonists on the LAR, the regulation of TRPV4 downstream mediator, ATP, was evaluated in a time-course experiment of the rat OVA model. Extracellular ATP levels were found elevated in the BAL of OVA-sensitised rats after OVA challenge, suggesting that pathways involving the release of ATP in the airways are indeed upregulated in the model, and by extent that TRPV4 upregulation could participate in this elevation.

Nevertheless, this increase in ATP levels was found at 6 h but not at earlier timepoints of 1 and 2 h post-challenge, while the LAR peaks at 3 h in this model. In human, ATP levels were found elevated at 24 h but not at 10 min post-allergen challenge in BAL fluid from mild atopic asthmatics (Idzko et al. 2007). This discrepancy between the rise in ATP levels and the timeframe of the LAR may be due to the fact that a certain level of ATP needs to accumulate in the airways before being detected, given that BAL fluid is a diluted version of the fluid lining the airways and measured levels of ATP were close to the detection limit of the assay. More importantly, it is unsure whether ATP levels assessed in BAL fluid would accurately reflect the amount of extracellular ATP released in the airways during the LAR, for the following reasons: 1) ATP can be released upon necrosis/apoptosis and ATP levels were assessed after sacrificing the animals; 2) ATP can be released upon mechanical stimuli and cell lysis and thus could be released during BAL retrieving and sample processing; 3) ATP is quickly degraded by ectonucleotidases (ATPases) present in the extracellular milieu (Gorman, Feigl, and Buffington 2007) and therefore local elevations of ATP in the airways may not be captured in BAL fluid. In addition, red blood cells and platelets exhibit a high content of ATP (Gorman, Feigl, and Buffington 2007) and therefore a difference in blood contamination between BAL samples from OVA-challenged rats compared to SAL-challenged rats could result in a

difference in ATP levels. The layout of the experiment could also be argued, as performing multiple Mann-Whitney tests can increase the risk of false positives.

Following the measure of ATP levels, the selective TRPV4 and P2X3-P2X2/3 antagonists were tested against the LAR in the rat OVA model. Both the TRPV4 antagonist GSK2193874 and P2X3-P2X2/3 antagonist AF-353 significantly prevented the late increase in Penh after OVA challenge in OVA-sensitised rats. These doses of TRPV4 and P2X3-P2X2/3 antagonists did not inhibit TRPV1-induced nerve firing nor cough in guinea pigs, confirming their selectivity *in vivo* against TRPV1 channels (Bonvini et al. 2016). Therefore, these findings supported the hypothesis that TRPV4 and P2X3-P2X2/3 receptors could be one of the drivers of the LAR in the rat OVA model.

As described in previous chapters, P2X3-P2X2/3 receptors are potent activators of airway sensory nerves (Kwong et al. 2008; Nassenstein et al. 2010; Weigand, Ford, and Undem 2012; Bonvini et al. 2016; Pelleg et al. 2019) and have been highly involved in eliciting cough in guinea pigs, another airway reflex (Bonvini et al. 2016; Pelleg et al. 2019). These receptors also seem important in human airway reflexes, as the P2X3-P2X2/3 antagonist AF-219 reduced daytime objective cough frequency in patients with refractory chronic cough with an unprecedented efficacy in a proof of concept study (Abdulqawi et al. 2015). In the literature, P2X3 expression seems restricted to sensory neurons (Burnstock 2007). Indeed, the receptor has not been found in airway epithelial cells (A. L. Taylor et al. 1999; Collo et al. 1996), airway smooth muscle cells (Collo et al. 1996; Gui et al. 2011), immune cells such as lung mast cells (Bradding et al. 2003; Flajolet et al. 2019), $CD4^+$ T cells, B cells (Woehrle et al. 2010; Adrian et al. 2000; Sluyter, Barden, and Wiley 2001) nor eosinophils (Wright et al. 2016). Therefore, any effect of the P2X3-P2X2/3 antagonist was likely due to airway sensory nerves blockade, thereby highlighting a role for airway sensory nerves activation in the LAR in the rat OVA model.

Results from **Chapter 3** and published report (Bonvini et al. 2016) provided evidence that TRPV4 activates airway sensory nerves via the release of ATP acting on P2X3-P2X2/3 receptors. Although the causal link between TRPV4 and P2X3-P2X2/3 receptors cannot be ascertained in the LAR, findings from this chapter favoured the hypothesis that TRPV4 could drive the LAR via activating a P2X3-P2X2/3-mediated nerve reflex. In addition, this notion of the TRPV4-P2X3 axis driving a nerve reflex has already been demonstrated in guinea pigs in the context of cough, where TRPV4 agonist GSK1016790A-induced cough was blocked by the P2X3-P2X2/3 antagonist AF-353 (Bonvini et al. 2016). This TRPV4-P2X3 axis has also been involved in mediating micturition reflex, with the TRPV4 agonists GSK1016790A and 4αPDD increasing rat bladder voiding and the effect being blocked by the P2X3-P2X2/3

antagonists TNP-ATP and A-317491 and by anaesthesia. In the same model, the TRPV4 agonist GSK1016790A elicited bladder afferents firing, with the signal being blocked by the P2X3-P2X2/3 antagonist TNP-ATP (Birder et al. 2007; Aizawa et al. 2012).

It is intriguing that two distinct TRP channels, TRPV4 and TRPA1, could be involved in the LAR in the same rat OVA model by activating airway sensory nerves. Indeed, the TRPA1 antagonist HC-030031, but not the TRPV1 antagonist JNJ-17203212, diminished the LAR in the rat OVA model in the laboratory (Raemdonck et al. 2012). TRPA1 is found directly expressed on airway sensory neurons and is known to fire C-fibres in guinea pigs, in contrast to TRPV4 which seems to indirectly activate Aδ-fibres (Adcock et al. 2014; Wortley et al. 2016). Both TRPA1 and TRPV4 can be stimulated upon PAR2 activation, a receptor which can be activated by mast cell degranulation products that are released upon allergen challenge (Dai et al. 2007; Grant et al. 2007; Poole et al. 2013; Grace et al. 2014). Therefore, it may be that TRPA1 and TRPV4 pathways are both activated after allergen challenge and interact. In fact, studies in anaesthetised guinea pigs suggested that baseline cholinergic tone is dependent on intrapulmonary Aδ-fibres while C-fibre activation enhances this cholinergic output by releasing neuropeptides centrally (Kesler and Canning 1999; Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002b). By activating C-fibres, TRPA1 may amplify the TRPV4-P2X3 Aδ-nerve signal and ultimately enhance cholinergic tone. Overall, the fact that TRPA1, TRPV4 and P2X3-P2X2/3 antagonists all diminished the LAR strongly supports the hypothesis that airway sensory nerve activation and an efferent reflex could cause the LAR, at least in this rat model.

Perhaps surprisingly, the TRPV4 antagonist GSK2193874 and P2X3-P2X2/3 antagonist AF-353 also reduced the airway inflammation associated with the late bronchospasm, with a reduction in the number of eosinophils and neutrophils in BAL fluid. As previously mentioned, the LAR is classically presented as a result of inflammatory cell recruitment, most notably eosinophils, in the airways (Gauvreau, El-Gammal, and O'Byrne 2015). The effect of TRPV4 and P2X3-P2X2/3 blockade on airway inflammation therefore challenged the idea of an LAR caused by a nerve reflex rather than by recruited inflammatory cells. However, the link between eosinophils recruitment and the late bronchospasm remains unclear in the literature. As previously explained, depletion of eosinophils with anti-IL-5 antibodies did not attenuate the LAR (Leckie et al. 2000) and eosinophils influx into the airways was reported to peak later than the LAR in atopic asthmatics (Lommatzsch et al. 2006). In the rat OVA model used in this thesis, eosinophils were found to accumulate in the lungs at 24 h post-challenge while the LAR peaks at 3 h (S. L. Underwood et al. 2002). Recent studies from the group also found that repeated OVA challenges over a period of 5 weeks consistently elicited LAR responses

whereas the magnitude of eosinophilic inflammation declined after each challenge (Flajolet et al. 2018), questioning the importance of eosinophils recruitment to elicit the LAR.

Nonetheless, the observation that TRPV4 blockade protected against allergen-induced inflammation may not be surprising when considering its indirect mechanism of action. Indeed, TRPV4 seems to exert its actions via the release of ATP and the latter can activate a wide range of purinergic receptors other than P2X3-P2X2/3 (North 2002). As such, P2X1, P2X4 and P2X7 receptors are highly expressed in immune cells and promote innate and adaptive immunity in preclinical models (Junger 2011). In T cell lines, P2X1, P2X4 and P2X7 silencing inhibited TCR-induced T cell activation (Woehrle et al. 2010). In allergic airway disease models, one group showed that degradation of ATP by apyrase, P2X4 deficiency or blockade by the P2X4 antagonist 5-BDBD, P2X7 deficiency or blockade by the P2X7 antagonist KN62 all reduced airway inflammation and T_H2 cytokines production in mouse OVA and HDM models. In these models, *P2x4*$^{-/-}$ and *P2x7*$^{-/-}$ DCs had an impaired T_H2-priming capacity (Idzko et al. 2007; Müller et al. 2011; Zech et al. 2016). In human, children bearing variants of *P2X7* with low pore function presented with less risk of developing asthma (Manthei et al. 2012). It is therefore conceivable that TRPV4 could exert pro-inflammatory effects via other receptors than P2X3-P2X2/3, such as P2X1, P2X4 or P2X7. Indeed, TRPV4 has been shown to promote inflammation in a preclinical COPD model, with *Trpv4*$^{-/-}$ mice exhibiting less ATP, IL-1β and neutrophilic inflammation in their airways upon cigarette smoke challenge, a model dependent on the P2X7-inflammasome pathway (Baxter et al. 2014). TRPV4 might be expressed on T cells and DCs (Bertin et al. 2014; Majhi et al. 2015; Naert et al. 2019), highlighting a possible role in adaptive immunity, however most of TRPV4 expression so far has been depicted in structural cells such as epithelial, airway smooth muscle and vascular cells and macrophages (D. F. Alvarez et al. 2006; Baxter et al. 2014; Jia et al. 2004; McAlexander et al. 2014; Rahaman et al. 2014; Fantozzi et al. 2003).

In fact, TRPV4 pro-inflammatory effects remain unclear in atopic airway disease, with only two reports in the literature. In a *D. farinae* model, *Trpv4*$^{-/-}$ mice had reduced neutrophilic inflammation and airway remodelling, yet eosinophilic inflammation, T_H2 cytokines production and bronchoconstriction responses were not assessed (Gombedza et al. 2017). In another group, *Trpv4*$^{-/-}$ mice exhibited similar levels of airway inflammation including eosinophils, T_H2 cytokines and AHR than wild-type mice in an acute OVA model, concluding of a dispensable role for TRPV4 in the development of allergic airway disease (Palaniyandi et al. 2019). These findings warrant further investigations into the role of TRPV4 in promoting inflammation in airway disease.

More surprising was the reduction of airway inflammation by the P2X3-P2X2/3 antagonist AF-353 in the rat OVA model. As P2X3 receptors expression seems restricted to airway sensory neurons (Burnstock 2007), this result may in fact suggest that nerve activation contributes to airway inflammation. Supporting this hypothesis, ablating airway nerves via vagotomy or targeted denervation of $Na_v1.8^+$ neurons has been associated with reduced airway inflammation in canine and murine models of allergic airway inflammation (R. Liu et al. 2014; S. Talbot et al. 2015). It has been described that nerves could contribute to driving inflammation by two mechanisms: neuropeptides and ACh-mediated inflammation. Indeed, airway sensory nerves can locally release neuropeptides such as the tachykinins substance P and neurokinin A (NKA). These peptides have been shown to promote the activation and recruitment of inflammatory cells, especially in guinea pigs (Sébastien Talbot, Foster, and Woolf 2016), and substance P has been found elevated in the airways of asthmatic patients after allergen challenge (Nieber et al. 1992). However, the role of tachykinins remains uncertain in asthma as tachykinin receptors antagonists have not shown efficacy in clinical trials (Boot et al. 2007). Alternatively, preclinical studies pointed out a pro-inflammatory role for ACh as the two anticholinergics tiotropium and aclidinium reduced eosinophilic inflammation in murine and guinea pig allergic airway disease models (Ohta et al. 2010; John-Schuster et al. 2017; Damera et al. 2010). It is uncertain how ACh promotes these effects, although studies have proposed that ACh enhances DCs priming of T_H2 responses (Gori et al. 2017; 2019). Further studies are needed to investigate how TRPV4 and P2X3-P2X2/3 receptors promote airway inflammation and how this relates to causing bronchospasm.

To conclude, results from this chapter suggest that both TRPV4 and P2X3-P2X2/3 receptors are involved in driving the LAR in the rat OVA model, supporting the hypothesis that a TRPV4-P2X3 nerve reflex could drive the LAR.

5 Investigating if the activation of TRPV4 triggers bronchospasm in naïve rats

5.1 Rationale

Anaesthesia and a LAMA blocked the LAR in the rat OVA model, suggesting that allergen challenge activates airway nerves and induces a cholinergic reflex leading to the LAR (Raemdonck et al. 2012). In **Chapter 3**, the TRPV4 agonist GSK1016790A depolarised vagal nerves isolated from naïve rats and this seemed to be mediated via the release of ATP through Panx1 channels and the activation of P2X3-P2X2/3 receptors, replicating findings in guinea pig and human vagal nerves (Bonvini et al. 2016). Upon these observations, it was hypothesised that TRPV4 could be a driver of the nerve reflex leading to the LAR, by activating P2X3-P2X2/3 receptors on airway sensory nerves and triggering a cholinergic reflex.

Supporting this hypothesis, results from **Chapter 4** showed that both a TRPV4 and a P2X3-P2X2/3 antagonist inhibited the LAR in the rat OVA model. While these findings made a strong case for a role of the TRPV4-P2X3 axis in activating afferent nerves in the LAR, these experiments did not ascertain whether it was involved in the LAR via triggering a cholinergic reflex. For this purpose, this chapter aimed to investigate the ability of the TRPV4-P2X3 axis to elicit a cholinergic reflex bronchospasm, using a pharmacological approach in naive Brown Norway rats.

5.2 Hypothesis

Activation of the TRPV4-P2X3 afferent nerve axis triggers a cholinergic reflex bronchospasm in naïve rats.

5.3 Aims

- Investigate whether TRPV4 activation can elicit bronchospasm in conscious naïve rats.
- Study whether TRPV4-induced bronchospasm is mediated via the activation of airway sensory nerves via P2X3-P2X2/3, using the selective P2X3-P2X2/3 antagonist.
- Study whether TRPV4-induced bronchospasm is mediated via the activation of a cholinergic reflex, using the LAMA tiotropium.
- Investigate if TRPV4 can provoke local bronchospasm independently from a nerve reflex.

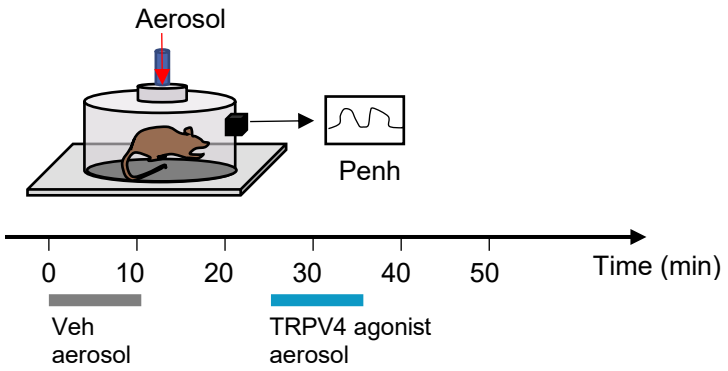
5.4 Methods

5.4.1 Effect of TRPV4 on Penh in conscious naive rats

The ability of TRPV4 activation to induce reflex bronchospasm was tested in conscious naïve rats by recording Penh using FWBP as described in **2.6.1**.

5.4.1.1 TRPV4 agonist Penh concentration-response in naïve rats

The ability of TRPV4 to induce bronchospasm was firstly tested by performing a concentration-response of the TRPV4 agonist GSK1016790A. Conscious naïve rats were placed in FWBP chambers. After establishing a 5-min baseline, rats were challenged with the vehicle (1% EtOH in saline) aerosolised for 10 min and Penh recorded for 20 min. After a 5-min recovery period, rats were subsequently challenged with a single dose of the TRPV4 agonist GSK1016790A (0.3, 3, 10 or 30 µg/ml corresponding to 0.46, 4.57, 15.25 and 45.70 µM) aerosolised for 10 min and Penh recorded for 20 min (**Figure 5-1**).

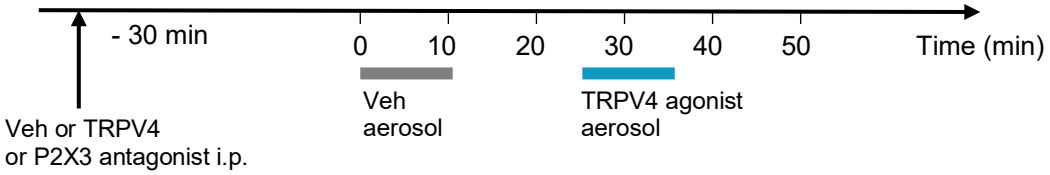


N	Challenge 1 Veh (1% EtOH)	Challenge 2 TRPV4 agonist (GSK1016790A)
4	Veh	0.3 µg/ml
4	Veh	3 µg/ml
4	Veh	10 µg/ml
4	Veh	30 µg/ml

Figure 5-1 Protocol of TRPV4 agonist concentration-response Penh study

5.4.1.2 Effect of TRPV4, P2X3 antagonists on TRPV4-induced Penh in naïve rats

The concentration of the TRPV4 agonist GSK1016790A eliciting a submaximal Penh increase (10 µg/ml or 15.25 µM) was selected and used in the study with the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or its vehicle (6% HP-β-cyclodextrin in saline, i.p.) and the P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) or its vehicle (10% PEG400 in saline, i.p.) in conscious naïve rats. Antagonists were administered intraperitoneally 30 min prior to the experiment. Thereafter, conscious rats were placed in FWBP chambers and, after establishing a 5-min baseline, challenged with the vehicle for the TRPV4 agonist (1% EtOH in saline) aerosolised for 10 min and Penh recorded for 20 min. After a 5-min recovery period, rats were challenged with the TRPV4 agonist GSK1016790A (10 µg/ml) aerosolised for 10 min and Penh recorded for 20 min (**Figure 5-2**).



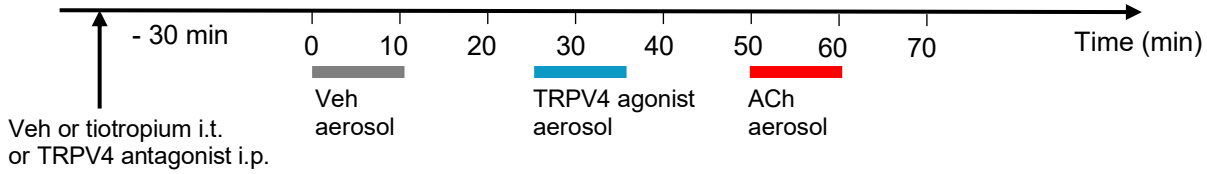
N	Treatment intraperitoneal		Challenges aerosol	
			Veh (1% EtOH)	TRPV4 agonist GSK1016790A (10 µg/ml)
8	Veh (6% HP-β-cyclodextrin)	Veh (10% PEG400)	Veh	TRPV4 agonist
8	TRPV4 antagonist GSK2193874 (300 mg/kg)	Veh (10% PEG400)	Veh	TRPV4 agonist
8	Veh (6% HP-β-cyclodextrin)	P2X3 antagonist AF-353 (30 mg/kg)	Veh	TRPV4 agonist

Figure 5-2 Protocol of TRPV4 and P2X3 antagonists Penh study

5.4.1.3 Effect of the LAMA tiotropium on TRPV4-induced Penh in naïve rats

The bronchospasm induced by the TRPV4 agonist GSK1016790A (10 µg/ml) was tested against the LAMA tiotropium (1 mg/kg, i.t.) or its vehicle (0.5% EtOH in saline, i.t.), and the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or its vehicle (6% HP-β-cyclodextrin in saline, i.p.) in conscious naïve rats. Tiotropium was administered intratracheally under isoflurane anaesthesia and the TRPV4 antagonist dosed intraperitoneally 30 min prior to the experiment. Thereafter, conscious rats were placed in FWBP chambers and, after establishing a 5-min baseline, challenged with the vehicle (1% EtOH in saline) for 10 min and Penh recorded for 20 min. After a 5-min recovery period, rats were challenged with the TRPV4

agonist GSK1016790A (10 µg/ml) for 10 min and Penh recorded for 20 min. After another 5-min recovery period, rats were challenged the muscarinic agonist ACh (16 mg/ml) as a positive control, aerosolised for 10 min and Penh recorded for 20 min (**Figure 5-3**).

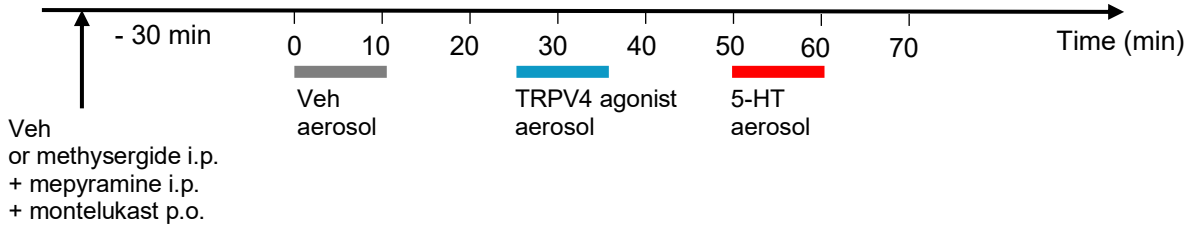


N	Treatment		Challenges aerosol		
	intraperitoneal	intratracheal	Veh (1% EtOH)	TRPV4 agonist GSK1016790A (10 µg/ml)	ACh (16 mg/ml)
8	Veh (6% HP-β-cyclodextrin)	Veh (0.5% EtOH)	Veh	TRPV4 agonist	ACh
8	Veh (6% HP-β-cyclodextrin)	Tiotropium (1mg/kg)	Veh	TRPV4 agonist	ACh
8	TRPV4 antagonist GSK2193874 (300 mg/kg)	Veh (0.5% EtOH)	Veh	TRPV4 agonist	ACh

Figure 5-3 Protocol of tiotropium Penh study

5.4.1.4 Effect of methysergide, mepyramine and montelukast on TRPV4-induced Penh in naïve rats

The concentration of the TRPV4 agonist GSK1016790A eliciting submaximal Penh increase (10 µg/ml) was tested against the combination of 5-HT receptor (5-HTR) antagonist methysergide (10 mg/kg, i.p.), histamine H1 receptor (H1R) antagonist mepyramine (10 mg/kg, i.p.) and CysLTs receptor 1 (CysLTR1) antagonist montelukast (30 mg/kg, p.o.) or their vehicle (saline, i.p. and methylcellulose 0.5% Tween80 0.2% in saline, p.o.) in conscious naïve rats. Antagonists were administered intraperitoneally or via oral gavage 30 min prior to the experiment. Thereafter, conscious rats were placed in FWBP chambers and, after establishing a 5-min baseline, challenged with the vehicle (1% EtOH in saline) for 10 min and Penh recorded for 20 min. After a 5-min recovery period, rats were challenged with the TRPV4 agonist GSK1016790A (10 µg/ml) for 10 min and Penh recorded for 20 min. After another 5-min recovery period, rats were challenged with the positive control 5-HT (8 mg/ml) aerosolised for 10 min and Penh recorded for 20 min (**Figure 5-4**).



N	Treatment		Challenges aerosol		
	intraperitoneal	oral gavage	Veh (1% EtOH)	TRPV4 agonist GSK1016790A (10 µg/ml)	5-HT (8 mg/ml)
8	Veh (saline)	Veh (methylcellulose 0.5% Tween80 0.2%)	Veh	TRPV4 agonist	5-HT
8	Methysergide, Mepyramine (10 mg/kg)	Montelukast (30 mg/kg)	Veh	TRPV4 agonist	5-HT

Figure 5-4 Protocol of methysergide, mepyramine, montelukast Penh study

5.4.1.5 Selection of antagonist doses in Penh studies

The doses of TRPV4 and P2X3-P2X2/3 antagonists were chosen based on dose-response studies in guinea pigs as previously described (Bonvini et al. 2016). Tiotropium dose was chosen as the dose eliciting maximum blockade of ACh-induced bronchospasm in Brown Norway rats in previous studies (unpublished). Doses of methysergide, mepyramine and montelukast were established as the doses eliciting maximum blockade of the effect provoked by their respective agonist 5-HT, histamine or LTD_4 in Brown Norway rats (Hele et al. 2001).

5.4.1.6 Data analysis of Penh studies

Penh data were averaged every min and plotted over time to calculate Penh AUC. Statistical analysis was performed on Penh AUCs. Non-parametric Wilcoxon tests compared responses between agonist and paired vehicle challenge. Non-parametric Mann-Whitney tests compared responses between antagonist and vehicle treatment.

5.4.2 Effect of TRPV4 on intratracheal pressure in an anaesthetised rat

The ability of TRPV4 to elicit local bronchospasm independently from a nerve reflex was tested *in vivo* using the single fibre preparation described in **2.6.2**. in an anaesthetised naïve rat.

A naïve rat was anesthetised, paralysed, artificially ventilated and the central ends of the two vagal nerve trunks were cut (bilateral cervical vagotomy) to inhibit reflex bronchospasm. After establishing baseline, the vehicle (1% EtOH in saline) was aerosolised into the rat trachea for 1 min. After 10 min, the TRPV4 agonist GSK1016790A (100 ng/ml or 152 nM) was aerosolised for 1 min. Intratracheal pressure (cm H_2O) was monitored to assess bronchospasm via a pressure transducer connected to the tracheal cannula.

Data were plotted as intratracheal pressure (cm H_2O) over time. No statistical analysis was performed as only N=1 experiment was conducted, corresponding to the same experiment presented in **3.5.4**. where the TRPV4 agonist induced Aδ-fibre firing.

5.4.3 Effect of TRPV4 activation on isolated trachea contraction

The ability of TRPV4 to contract naïve rat extrapulmonary airways was tested *in vitro* using organ bath preparations of tracheas and main bronchi as described in **2.6.3**. isolated from naïve rats.

Rat tracheas and main bronchi segments were incubated with ACh (1 mM) to determine maximal contraction. After several washes and return to baseline tension, tissues were incubated with either the vehicle (0.1% DMSO) or the TRPV4 agonist GSK1016790A (100 nM). After 40 min, tissues were washed and ACh (1 mM) was reapplied to ensure tissue viability was not affected by drug treatment. Changes in isometric tension (mg) were monitored to assess tissue contraction. A first set of experiment was conducted in presence of the non-selective COX inhibitor indomethacin (10 µM). A second set of experiment compared the contraction in presence and absence of indomethacin.

The TRPV4 agonist concentration was chosen as the concentration eliciting submaximal contraction of human bronchi and guinea pig tracheas in concentration-response studies (McAlexander et al. 2014; Bonvini et al. 2020).

Data were expressed as the maximum tension (mg) elicited by a compound during the 40-min incubation. For statistical analysis, data were normalised to the maximal contraction elicited by ACh for each tissue segment and analysed using non-parametric Mann-Whitney tests comparing vehicle and agonist response.

5.4.4 Effect of TRPV4 on small airways contraction using precision-cut lung slices (PCLS)

The ability of TRPV4 to contract intrapulmonary airways was tested *in vitro* using precision-cut lung slices (PCLS) generated from naïve rats as described in **2.6.4**. On day 1 post-isolation, PCLS were incubated with ACh (1 mM) to select viable airways contracting more than 50% to ACh. Airways with a similar range of diameters and ACh responses were distributed among treatment groups. On day 2 post-isolation, pharmacological experiments were performed. Airway contraction was monitored using light microscopy.

5.4.4.1 TRPV4 agonist vs TRPV4, Panx1, P2X3 and P2X4 antagonists

Baseline images were taken of each identified airway. PCLS were incubated with the TRPV4 antagonist GSK2193874 (10 µM), P2X3-P2X2/3 antagonist AF-353 (10 µM), P2X4 antagonist 5-BDBD (50 µM), Panx1 blocker probenecid (1 mM) or vehicle (0.1% DMSO) for 20 min. Without washing, the TRPV4 agonist GSK1016790A (100 nM) or vehicle (0.1% DMSO) was added for 40 min. Images were obtained every 10 min. Without washing, ACh (1 mM) was added for 10 min to assess airway viability and maximum contractility at the end of the experiment.

5.4.4.2 ATP vs P2X4 antagonist

Baseline images were taken of each identified airway. Slices were incubated with the P2X4 antagonist 5-BDBD (50 µM) or vehicle (0.1% DMSO) for 10 min. Without washing, ATP (1 mM) or the vehicle (1% H_2O) was added to the slices for 10 min. Images were obtained 30 sec, 1 min, 2 min, 5 min, 10 min and 20 min after adding drugs. Without washing, ACh (1 mM) was added for 10 min to assess airway viability and maximum contractility at the end of the experiment.

5.4.4.3 Selection of agonist and antagonist concentrations in PCLS studies

TRPV4 agonist concentration was chosen as the concentration eliciting submaximal contraction of human bronchi and guinea pig tracheas (McAlexander et al. 2014; Bonvini et al. 2020). P2X3-P2X2/3, Panx1 and P2X4 antagonist concentrations were determined as the concentration eliciting maximum blockade of their agonist in concentration-responses studies on guinea pig isolated tracheas and vagal nerves (Bonvini et al. 2020; 2016). ATP concentration was chosen as the concentration eliciting submaximal contraction of guinea pig tracheas and rat airways (Mounkaäila, Marthan, and Roux 2005).

5.4.4.4 Data analysis of PCLS experiments

Contraction was plotted over time by measuring airway lumen area and calculating the percentage closure from baseline at each timepoint.

To analyse the contraction elicited by an agonist, the percentage closure was calculated from the baseline area captured right before agonist incubation. The maximum percentage closure elicited during agonist incubation was referred to as "% airway closure". To compare responses, this was normalised to the contraction elicited by final incubation with ACh (1 mM) and referred to as "% of ACh contraction". In all experiments, groups were set as the following (agonist/antagonist): Veh/Veh; Veh/Agonist; Antagonist/Agonist. Non parametric Mann-Whitney tests compared data between Veh/Veh and Veh/Agonist groups and between Veh/Agonist and Antagonist/Agonist groups.

The Spearman rank coefficient was calculated to evaluate the correlation between the magnitude of the contraction and the airway diameter.

5.4.4.5 Range of airway diameters and ACh responses assessed in PCLS experiments

Airways tested in this thesis exhibited internal diameters from 37 to 734 µm, with a mean of 193 µm (N=9 rats, n=15-53 airways/rat with n=268 total airways) as shown in (**Figure 5-5**). As the definition of small airways has not been determined for the rat species, the human definition was applied in this thesis. In human, small airways are defined as exhibiting an internal diameter inferior to 2mm which approximately corresponds to airways located below the 8th generation (Weibel 1963). In rats, airways below the 8th generation have been shown to exhibit internal diameters inferior to 1 mm (Yeh, Schum, and Duggan 1979). Given that airways assessed in this thesis presented diameters inferior to 1 mm, these were considered as rat small airways.

Airways tested in this thesis contracted to ACh (1 mM) on day 1 post-isolation with a percentage airway closure ranging from 51 to 100%, with a mean closure of 84% (N=9 rats, n=15-53 airways/rat with n=268 total airways) as shown in (**Figure 5-5**) and similar to published results (Bergner and Sanderson 2002a). A correlation was found between airway diameter and contraction to ACh: the smaller the airway, the more it contracted to ACh, replicating results found in rat airways with a stable ACh analogue (C. Martin, Uhlig, and Ullrich 1996).

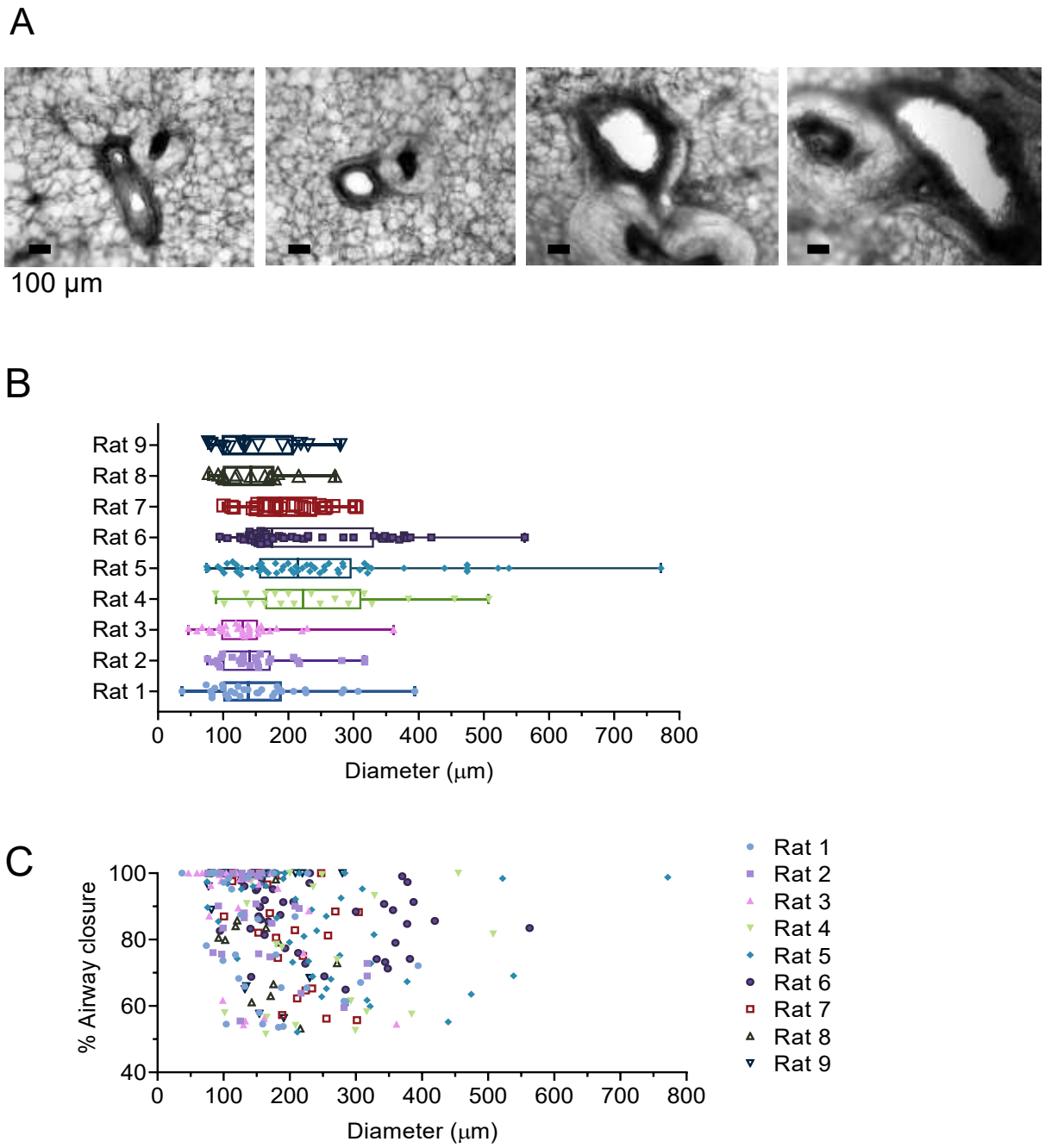


Figure 5-5 Range of airway diameters and ACh responses assessed in PCLS

PCLS were isolated from naïve rats and screened on day 1 post-isolation to select viable airways contracting > 50% to ACh (1 mM). These figures represent the range of diameters and ACh responses assessed with the PCLS technique. **(A)** Representative pictures of the types of airways assessed, scale bar = 100 µm. **(B)** Range of airway diameters obtained in PCLS experiments in this chapter. Data presented as a box plot with median as the middle line. **(C)** Relation between airway diameter and maximum contraction to ACh (1 mM). N=9 rats, n=15-53 airways/rat with n=268 total airways assessed in this chapter. Spearman coefficient r=-0.4164, $p<0.05$ between airway diameter and ACh contraction.

5.5 Results

5.5.1 Effect of the TRPV4 agonist on Penh in conscious naïve rats

To test the ability of TRPV4 to elicit reflex bronchospasm in naïve rats, experiments were conducted in conscious animals using Penh measured by FWBP. A non-cumulative concentration-response of the TRPV4 agonist GSK1016790A (0.3, 3, 10 and 30 µg/ml, corresponding to 0.46, 4.57, 15.25 and 45.7 µM) was firstly performed. Aerosolising the TRPV4 agonist seemed to increase Penh AUC compared to the vehicle (1% EtOH) however without reaching statistical significance (N=4 per group) (**Figure 5-6**).

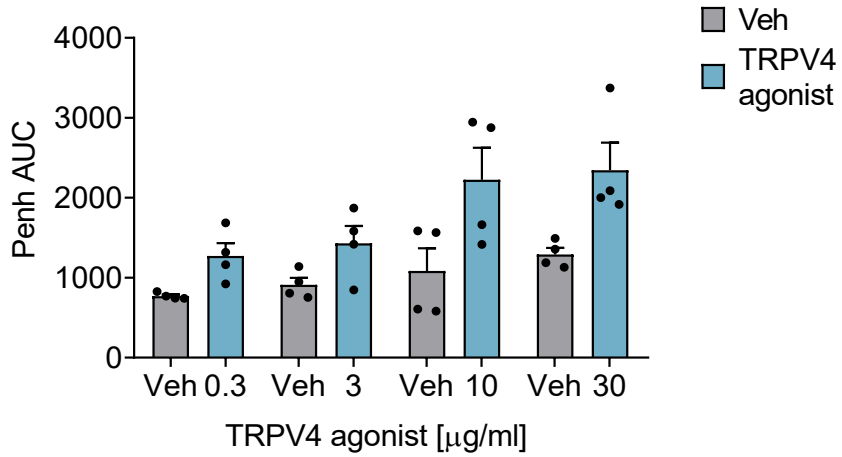


Figure 5-6 Effect of the TRPV4 agonist on Penh in naive conscious rats

Conscious naïve rats were challenged with the vehicle (1% EtOH) followed by a single concentration of the TRPV4 agonist GSK1016790A (0.3, 3, 10, 30 µg/ml corresponding to 0.46, 4.57, 15.25 and 45.70 µM) aerosolised for 10 min and Penh recorded for 20 min using FWBP. The graph represents the quantification of Penh AUC, N=4/group. Data presented as mean ± S.E.M. Wilcoxon test non-significant between agonist and paired vehicle challenges.

5.5.2 Effect of TRPV4 and P2X3 antagonists on TRPV4-induced Penh in naïve rats

The concentration of the TRPV4 agonist GSK1016790A that elicited a submaximal increase in Penh in the preliminary study was taken forward to antagonist studies. This time, aerosolising the TRPV4 agonist GSK1016790A at 10 µg/ml (or 15.25 µM) significantly increased Penh AUC compared to the vehicle (1% EtOH) (1646 ± 325.3 vs 2497 ± 404.4 Penh AUC) in naïve conscious rats (N=8 per group). The TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) significantly prevented this Penh increase compared to its vehicle (6% HP-β-

cyclodextrin) (2497 ± 404.4 vs 1523 ± 199.2 Penh AUC) (**Figure 5-7**). To investigate whether TRPV4 elicited bronchospasm via the activation of P2X3-P2X2/3 receptors, the P2X3-P2X2/3 antagonist AF-353 was tested in the same study. However, the P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) did not prevent TRPV4 agonist-induced Penh increase compared to its vehicle (10% PEG400) (2497 ± 404.4 vs 2746 ± 347.4 Penh AUC) (**Figure 5-7**).

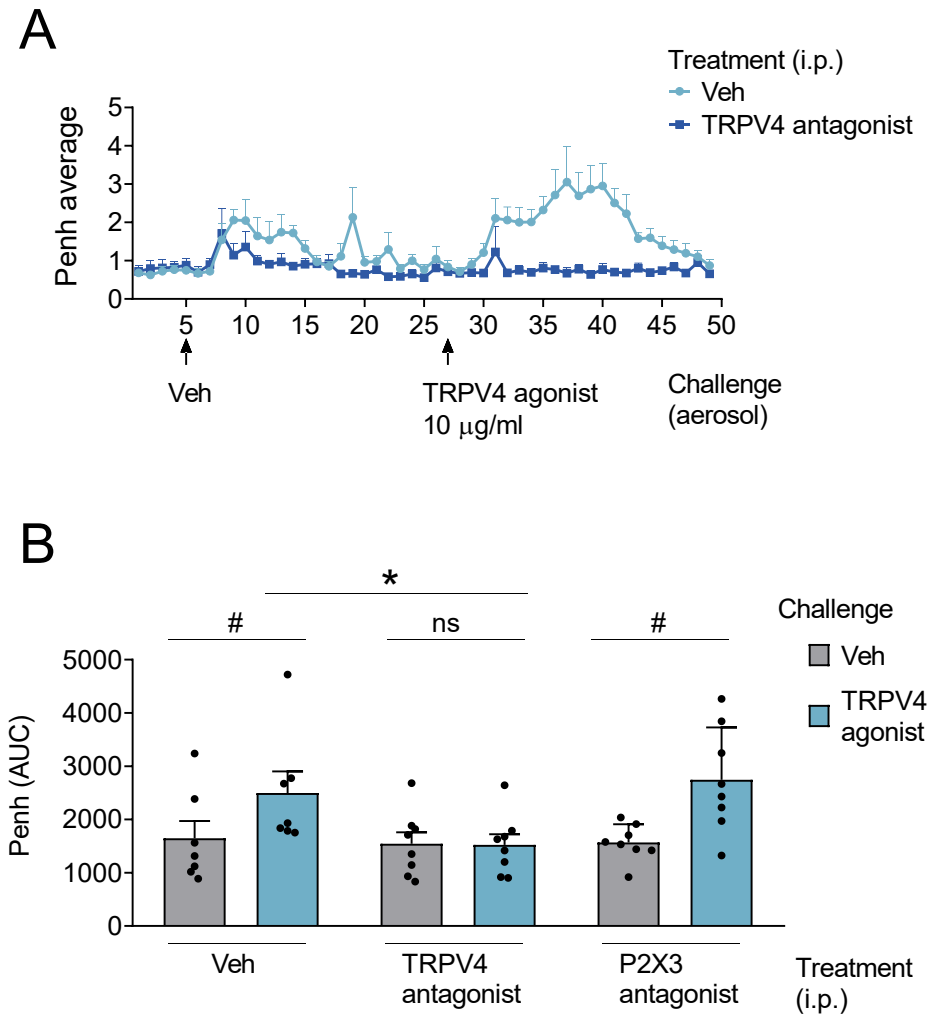


Figure 5-7 Effect of TRPV4 and P2X3 antagonists on TRPV4-induced Penh

Conscious naïve rats were dosed with the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.), P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) or vehicle (6% HP-β-cyclodextrin, i.p. and 10% PEG400, i.p.) 30 min before challenge with the vehicle (1% EtOH) followed by the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) aerosolised for 10 min and Penh recorded for 20 min after each challenge using FWBP. **(A)** Penh average over time. **(B)** Quantification of Penh AUC, N=8/group. Data presented as mean ± S.E.M. Wilcoxon test $^{\#}p<0.05$ agonist vs paired vehicle challenge. Mann-Whitney test *p<0.05 antagonist vs vehicle treatment.

5.5.3 Effect of tiotropium on TRPV4-induced Penh in naïve rats

To test the hypothesis that TRPV4 activation provoked a cholinergic reflex bronchospasm, the LAMA tiotropium was tested against TRPV4-induced Penh response in conscious naïve rats.

In this experiment, aerosolising the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) significantly increased Penh AUC compared to the vehicle (1% EtOH) (1470 ± 140.1 vs 2449 ± 330.9 Penh AUC) and this was prevented by the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) (1072 ± 59.62 vs 908.2 ± 91.52 Penh AUC). Tiotropium (1 mg/kg, i.t.) did not inhibit this increase in Penh (822.0 ± 92.53 vs 2771 ± 317.9 Penh AUC) (**Figure 5-8**).

Rats were subsequently challenged with ACh (16 mg/ml) as a positive control of tiotropium efficacy. Tiotropium inhibited the increase in Penh AUC caused by ACh compared to its vehicle (0.5% EtOH) (3041 ± 405.0 vs 1352 ± 201.3 Penh AUC) whereas the TRPV4 antagonist had no effect on ACh response (3041 ± 405.0 vs 3115 ± 301.2 Penh AUC) (**Figure 5-8**).

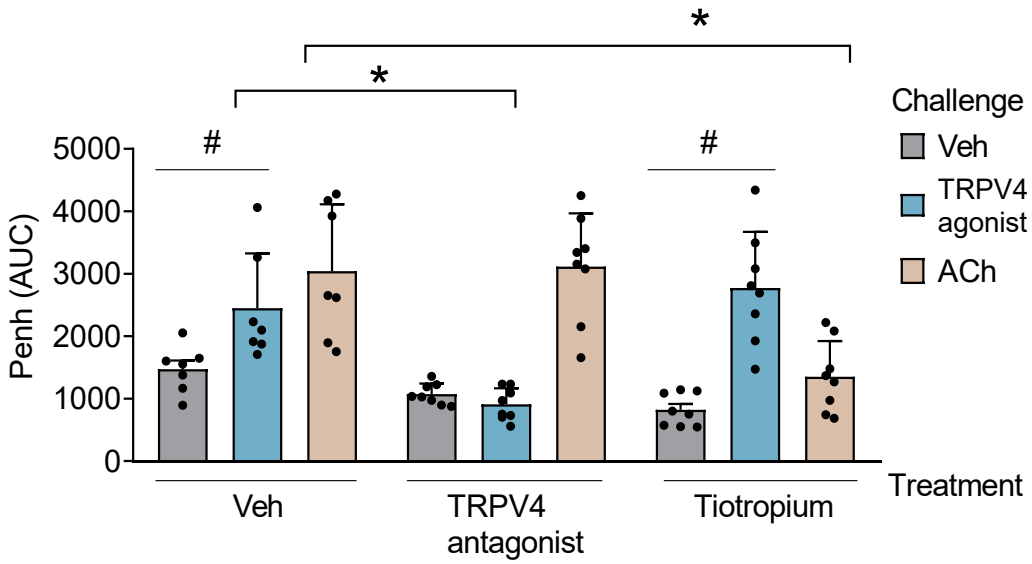


Figure 5-8 Effect of tiotropium on TRPV4-induced Penh

Conscious naïve rats were dosed with the LAMA tiotropium (1 mg/kg, i.t.) or TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or vehicle (0.5% EtOH, i.t. and 6% HP-β-cyclodextrin, i.p.) 30 min before challenge with the vehicle (1% EtOH) followed by the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) and by ACh (16 mg/ml) aerosolised for 10 min and Penh recorded for 20 min after each challenge using FWBP. The graph represents the quantification of Penh AUC, N=8/group. Data presented as mean ± S.E.M. Wilcoxon test $^{\#}p<0.05$ agonist vs paired vehicle challenge. Mann-Whitney test $^{*}p<0.05$ antagonist vs vehicle treatment.

5.5.4 Effect of TRPV4 on intratracheal pressure in an anaesthetised naïve rat

The observation that neither the P2X3-P2X2/3 antagonist nor the LAMA tiotropium inhibited the Penh response caused by the TRPV4 agonist unexpectedly suggested that TRPV4 provoked bronchospasm independently from a P2X3-mediated cholinergic reflex in naïve rats. In light of these results, further experiments tested the hypothesis that TRPV4 activation elicited local bronchoconstriction independently from a nerve reflex.

A naïve rat was anaesthetised, paralysed and both vagi disconnected from their central end to prevent reflex bronchospasm. Aerosolising the TRPV4 agonist GSK1016790A (100 ng/ml or 152 nM) for 1 min into the rat trachea was quickly followed by a short-lasting increase in intratracheal pressure compared to the vehicle (1% EtOH) (**Figure 5-9**).

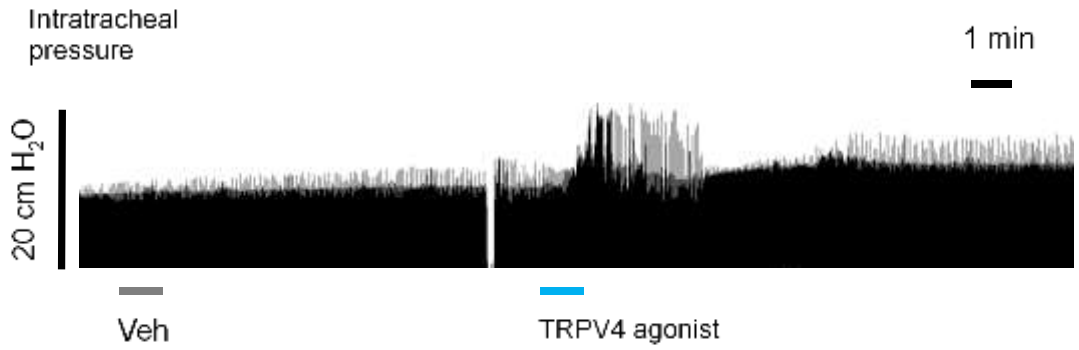


Figure 5-9 Effect of the TRPV4 agonist on local bronchospasm

A naïve rat was anaesthetised, paralysed and both vagal nerves disconnected from their central end to prevent reflex bronchospasm. The vehicle (1% EtOH) followed by the TRPV4 agonist GSK1016790A (100 ng/ml or 152 nM) were aerosolised for 1 min each directly into a tracheal cannula. Intratracheal pressure was assessed via a pressure transducer connected to the tracheal cannula. The figure shows the trace of intratracheal pressure (cm H_2O) over time, N=1 rat.

5.5.5 Effect of TRPV4 on isolated trachea and main bronchi contraction from naïve rats

As TRPV4 activation seemed to provoke bronchospasm in naïve rats independently from a nerve reflex, further studies were conducted *in vitro* on isolated airways to confirm these unexpected findings.

The effect of TRPV4 was tested on the contraction of extrapulmonary airways using organ bath preparations of tracheas and main bronchi generated from naïve rats. The TRPV4 agonist GSK1016790A (100 nM) did not provoke the contraction of tracheal nor main bronchi segments compared to the vehicle (0.1% DMSO) (**Figure 5-10**).

As per standard procedure, this experiment was conducted in the presence of the non-selective COX inhibitor indomethacin (10 µM) to prevent influences from prostanoids production that can be elicited by the dissection. To confirm that indomethacin was not masking a TRPV4 prostanoid-mediated contraction, another experiment compared TRPV4 agonist response in presence and absence of indomethacin. The presence of indomethacin did not affect the response (**Figure 5-10**).

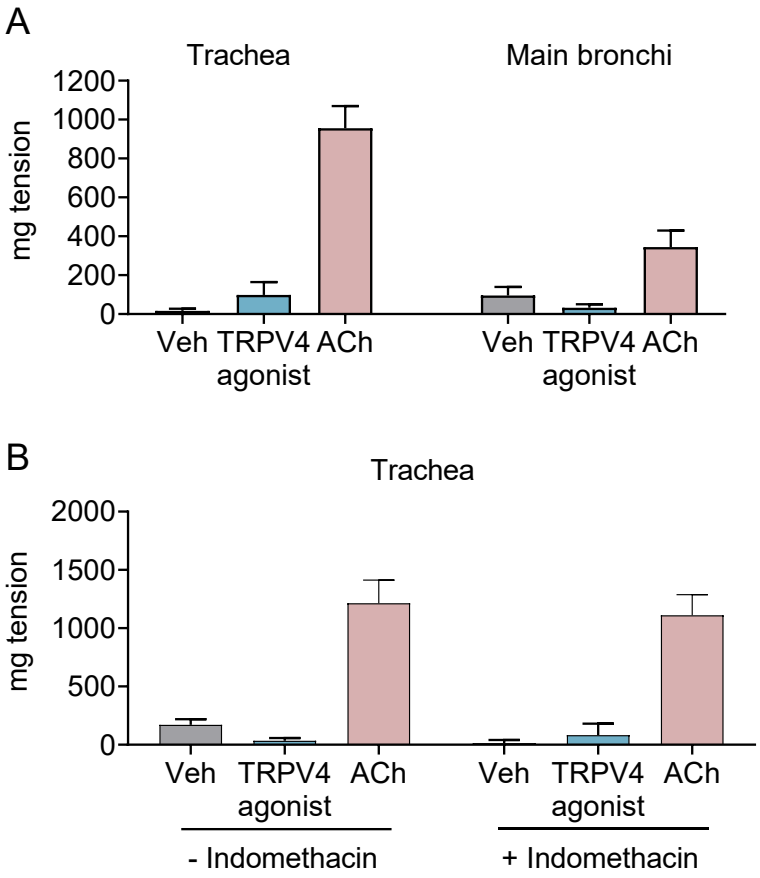


Figure 5-10 Effect of the TRPV4 agonist on trachea and main bronchi contraction

Rat isolated tracheal and main bronchi segments were incubated with ACh (1 mM) to determine viability and maximum contractility. After washing, segments were incubated with a single application of the vehicle (0.1% DMSO) or TRPV4 agonist GSK1016790A (100 nM) and contraction monitored for 40 min. **(A)** Contraction of tracheal and main bronchi segments in presence of indomethacin (10 μM), N=4 rats per group, n=1-2 tissue segments per rat. **(B)** Contraction of tracheal segments in another set of experiment, in absence (-) or presence (+) of indomethacin (10 μM), N=4 rats per group, n=1-2 tissue segments per rat. Combined ACh initial responses are represented for comparison. Data expressed as mean ± S.E.M. Mann-Whitney test non-significant between vehicle and agonist responses on data normalised to ACh response.

5.5.6 Effect of TRPV4 on small airways contraction in PCLS from naïve rats

Given that TRPV4 activation did not appear to contract rat tracheas nor main bronchi, it was hypothesised that TRPV4 provoked bronchospasm in naïve rats by engaging intrapulmonary airways. To study this hypothesis, the effect of the TRPV4 agonist was tested on small airways contraction using precision-cut lung slices (PCLS) generated from naïve rats.

The TRPV4 agonist GSK1016790A (100 nM) elicited the contraction of small airways from naïve rats with a maximum corresponding to 53% of ACh contraction, whereas the vehicle (0.1% DMSO) did not seem to provoke such effect (5.7 ± 2.2 vs 41.7 ± 3.7 % airway closure). This contraction was significantly inhibited by the TRPV4 antagonist GSK2193874 (10 µM) (41.7 ± 3.7 vs -3.6 ± 1.3 % airway closure) (**Figure 5-11**). Final ACh (1 mM) responses were not different between treatment groups.

The magnitude of TRPV4 agonist-induced contraction normalised to ACh seemed to negatively correlate with airway diameter, suggesting that, the smaller the airway, the more it contracted to TRPV4 stimulation (**Figure 5-12**).

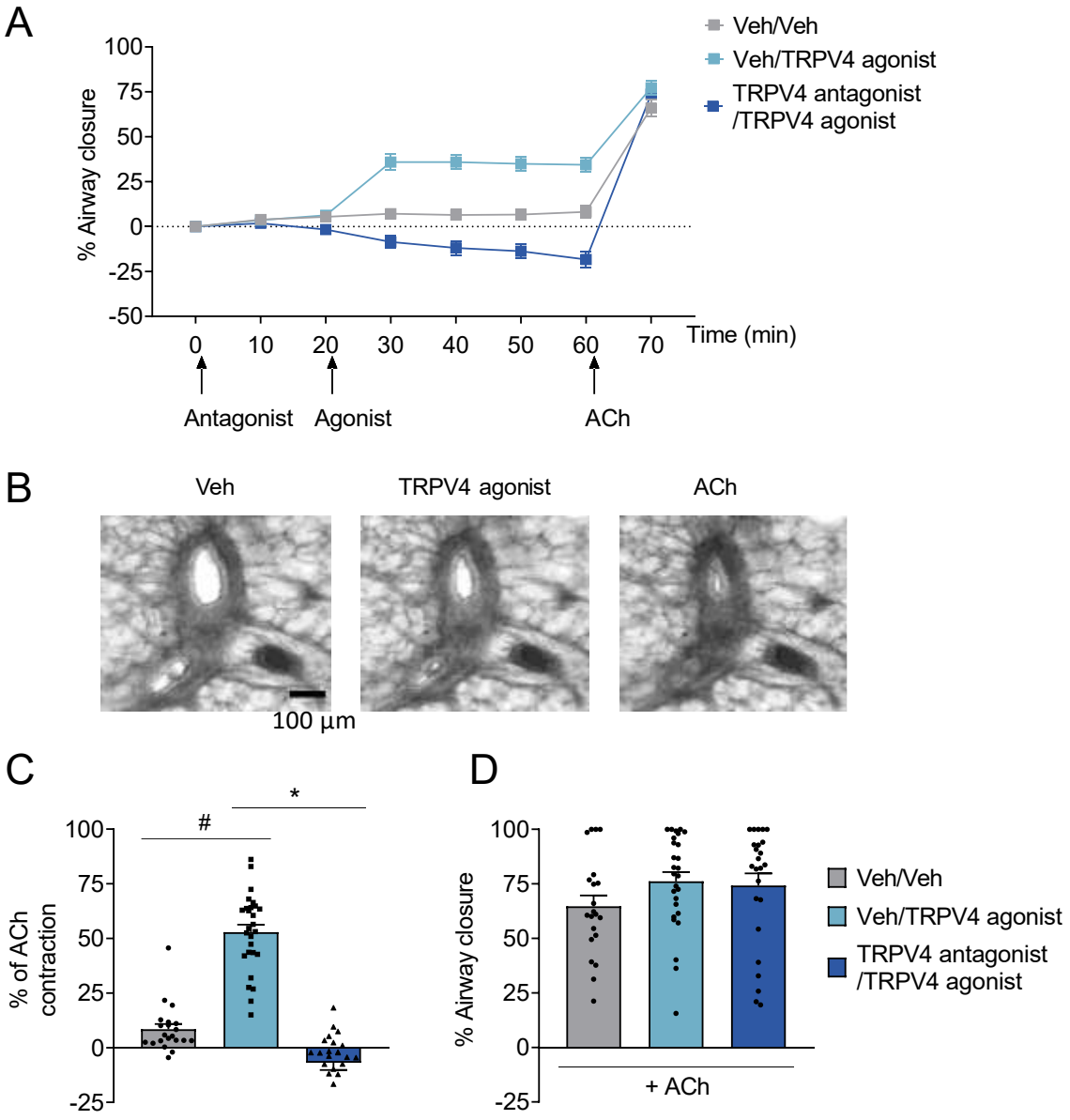


Figure 5-11 Effect of the TRPV4 agonist on small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or TRPV4 antagonist GSK2193874 (10 µM) for 20 min and challenged with the vehicle (0.1% DMSO) or TRPV4 agonist GSK1016790A (100 nM) for 40 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Representative pictures of an airway contracting to the TRPV4 agonist. **(C)** Quantification of the maximum contraction normalised to ACh response, Mann-Whitney test $^{\#}p<0.05$ Veh/Veh vs Veh/Agonist and $^{*}p<0.05$ Veh/Agonist vs Antagonist/Agonist. **(D)** Quantification of ACh final responses, Kruskal-Wallis test non-significant between treatment groups. Data presented as mean ± S.E.M. N=4 rats, n=3-12 airways/rat.

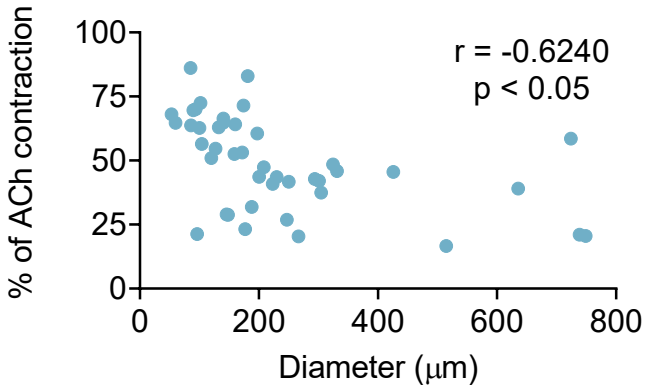


Figure 5-12 Correlation between airways diameter and contraction to TRPV4

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) for 20 min and challenged with the TRPV4 agonist GSK1016790A (100 nM) for 40 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. The graph represents the correlation between airway diameter and maximum contraction to the TRPV4 agonist normalised to ACh response. N=4 rats, n=3-12 airways/rat. Spearman correlation coefficient rank r=-0.6240, p<0.05.

5.5.7 Effect of the Panx1 blocker on TRPV4 and ATP-induced small airways contraction

To decipher the mechanism by which TRPV4 provoked small airways contraction, antagonist studies were performed on PCLS generated from naïve rats.

The Panx1 blocker probenecid (1 mM) was used to test if TRPV4 activation contracted rat small airways via the release of ATP through Panx1 channels. In this experiment, the TRPV4 agonist GSK1016790A (100 nM) provoked small airways contraction compared to its vehicle (0.1% DMSO) (8.0 ± 1.4 vs 35.5 ± 3.7 % airway closure) with a maximum corresponding to 42% of ACh contraction. This contraction was significantly reduced in presence of the Panx1 blocker probenecid (1 mM) (35.5 ± 3.7 vs 16.0 ± 2.4 % airway closure). At the end of the experiment, airways treated with the vehicles appeared to contract less to ACh (1 mM) than the other groups. The Panx1 blocker however did not seem to affect ACh response compared to its vehicle (**Figure 5-13**).

To confirm the hypothesis that ATP can provoke rat small airways contraction, the effect of incubating PCLS with ATP was tested in another experiment. ATP (1 mM) induced an immediate and short-lasting contraction with a duration of approximately 5 min compared to the vehicle (1% H_2O) (5.3 ± 1.8 vs 56.7 ± 3.8 % airway closure), with a maximum corresponding to 76% of ACh contraction. Final ACh (1 mM) responses were not different between treatment groups (**Figure 5-14**).

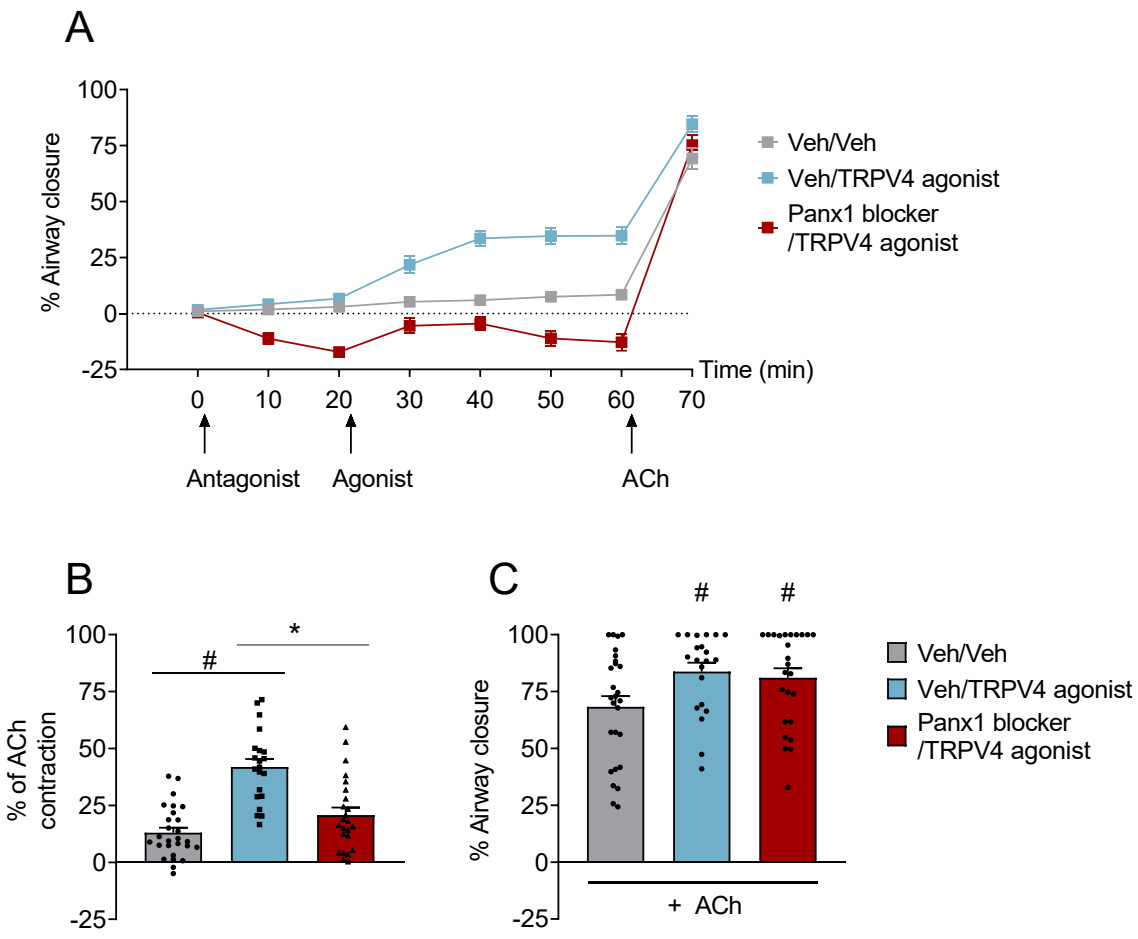


Figure 5-13 Effect of the Panx1 blocker on TRPV4-induced small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or Panx1 blocker probenecid (1 mM) for 20 min and challenged with the vehicle (0.1% DMSO) or TRPV4 agonist GSK1016790A (100 nM) for 40 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Quantification of maximum contraction normalised to ACh response, Mann-Whitney test $^{\#}p<0.05$ Veh/Veh vs Veh/Agonist and $^{*}p<0.05$ Veh/Agonist vs Antagonist/Agonist. **(C)** Quantification of ACh final responses, Kruskal-Wallis test significant between treatment groups, Mann-Whitney $^{\#}p<0.05$ vs Veh/Veh, non-significant between Veh/Agonist and Antagonist/Agonist. Data presented as mean ± S.E.M. N=5 rats, n=3-8 airways/rat.

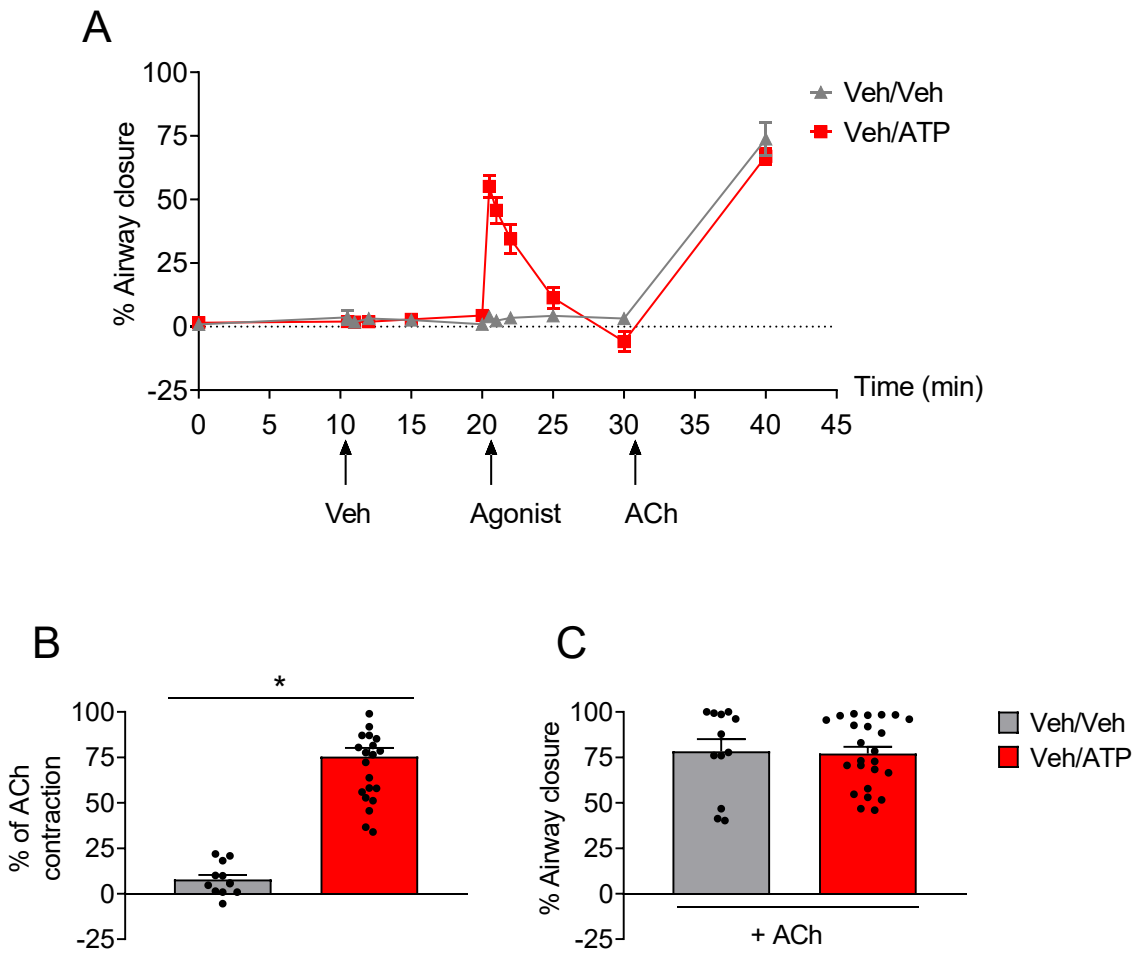


Figure 5-14 Effect of ATP on small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (1% H_2O) for 10 min and challenged with the vehicle (1% H_2O) or ATP (1 mM) for 10 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Quantification of maximum contraction normalised to ACh response, Mann-Whitney test *p<0.05. **(C)** Quantification of ACh final responses, Mann-Whitney test non-significant. Data presented as mean ± S.E.M. N=4 rats, n=3-7 airways/rat.

5.5.8 Effect of the P2X3 antagonist on TRPV4-induced small airways contraction

Given previous results, it was hypothesised that TRPV4 activation provoked small airways contraction via the release of ATP through Panx1 channels and the activation of P2X receptors. To test this hypothesis, P2X antagonists were used, starting with the P2X3-P2X2/3 antagonist AF-353.

In this experiment, the TRPV4 agonist GSK1016790A (100 nM) induced rat small airways contraction compared to its vehicle (0.1% DMSO) (2.9 ± 3.9 vs 33.2 ± 2.0 % airway closure) with a maximum corresponding to 40% of ACh contraction. This contraction was not inhibited by the P2X3-P2X2/3 antagonist AF-353 (10 µM) (33.2 ± 2.0 vs 47.0 ± 5.8 % airway closure). Final ACh (1 mM) responses were similar between groups (**Figure 5-15**).

5.5.9 Effect of the P2X4 antagonist on TRPV4 and ATP-induced small airways contraction

In human and guinea pigs, TRPV4 activation induces large airways contraction via the release of ATP activating P2X4 receptors (McAlexander et al. 2014; Bonvini et al. 2020). Therefore, the P2X4 antagonist 5-BDBD was used to study if a similar mechanism was involved in rat small airways contraction.

In this experiment, the TRPV4 agonist GSK1016790A (100 nM) induced rat small airways contraction compared to its vehicle (0.1% DMSO) (2.9 ± 2.3 vs 31.6 ± 2.7 % airway closure) with a maximum corresponding to 36% of ACh contraction. This contraction was inhibited by the P2X4 antagonist 5-BDBD (50 µM) (31.6 ± 2.7 vs 6.0 ± 4.3 % airway closure) (**Figure 5-16**). At the end of the experiment, airways treated with the P2X4 antagonist 5-BDBD displayed a significantly lower magnitude of ACh (1 mM) response compared to other groups, potentially indicating a non-specific or toxic effect. This difference was not due to unequal assignment of airways prior to the start of the experiment, as ACh responses on day 1 post-isolation were not different between groups (data not shown).

As a positive control, the P2X4 antagonist 5-BDBD was tested against ATP-mediated contraction. In this experiment, ATP (1 mM) induced a short-lasting small airways contraction (2.4 ± 1.8 vs 55.7 ± 4.7 % airway closure) with a maximum corresponding to 72% of ACh contraction. This contraction was not blocked by the P2X4 antagonist 5-BDBD (50 µM) (55.7 ± 4.7 vs 57.6 ± 9.6 % airway closure) (**Figure 5-17**).

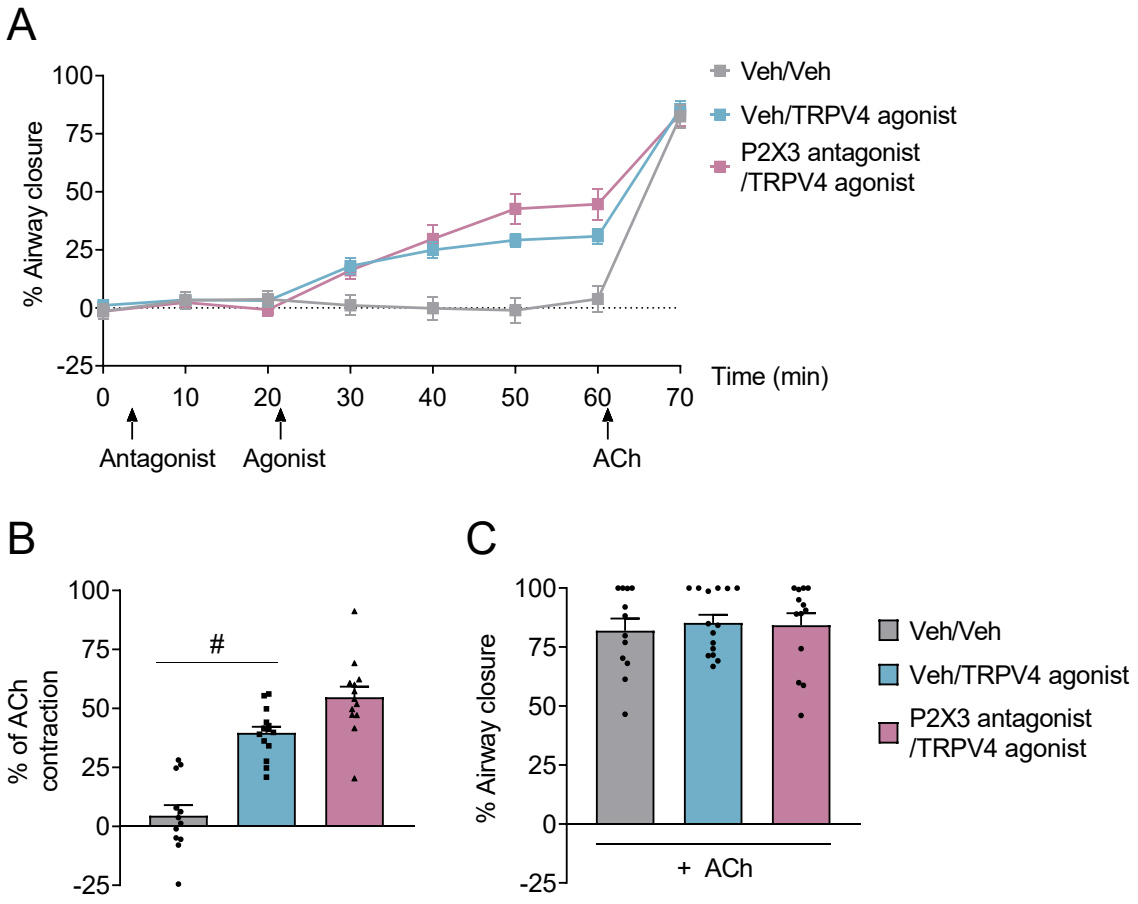


Figure 5-15 Effect the P2X3 antagonist on TRPV4-induced small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or P2X3-P2X2/3 antagonist AF-353 (10 μM) for 20 min and challenged with the vehicle (0.1% DMSO) or TRPV4 agonist GSK1016790A (100 nM) for 40 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Quantification of maximum contraction normalised to ACh response, Mann-Whitney test $^{\#}p<0.05$ Veh/Veh vs Veh/Agonist, non-significant Veh/Agonist vs Antagonist/Agonist. **(C)** Quantification of ACh final responses, Kruskal-Wallis non-significant. Data presented as mean ± S.E.M. N=3 rats, n=3-6 airways/rat.

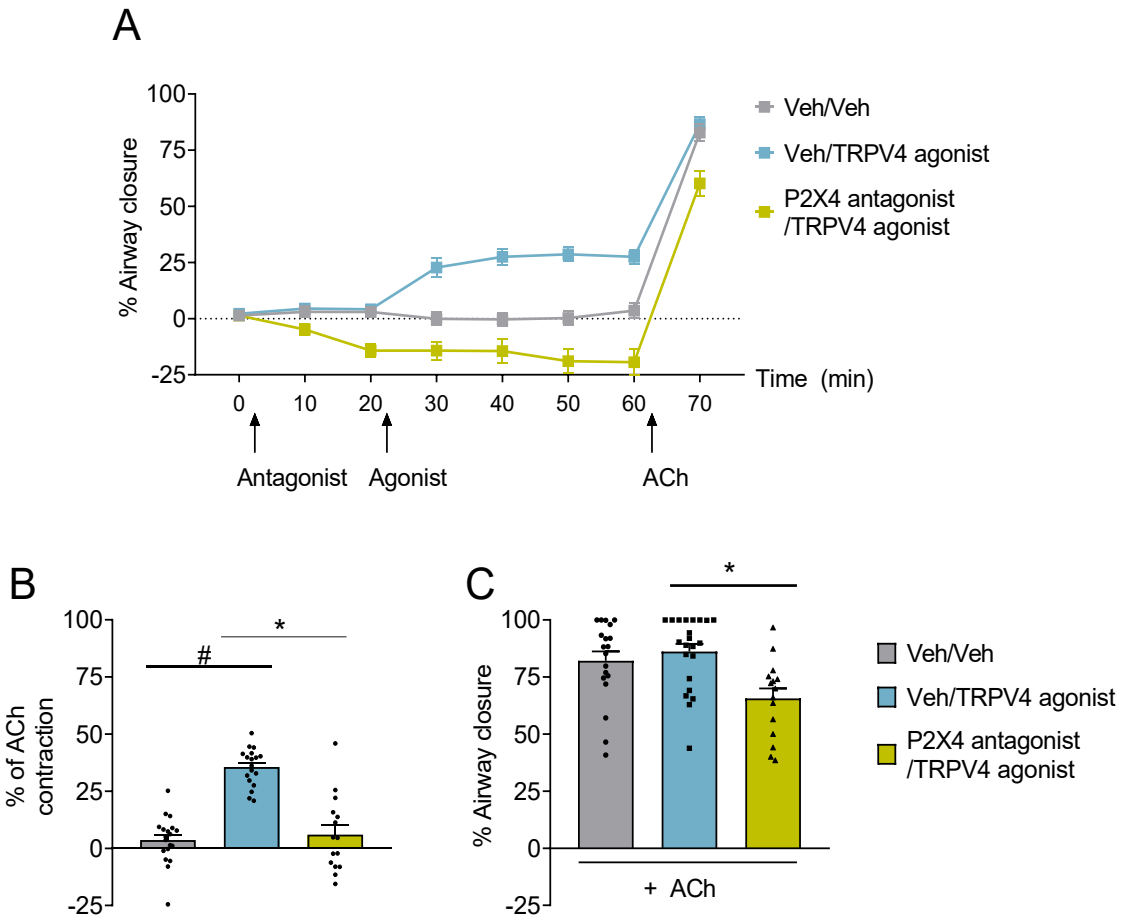


Figure 5-16 Effect of the P2X4 antagonist on TRPV4-induced small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or TRPV4 antagonist 5-BDBD (50 μM) for 20 min and challenged with the vehicle (0.1% DMSO) or TRPV4 agonist GSK1016790A (100 nM) for 40 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Quantification of maximum contraction normalised to ACh response, Mann-Whitney test $^{\#}p<0.05$ Veh/Veh vs Veh/Agonist and $^{*}p<0.05$ Veh/Agonist vs Antagonist/Agonist. **(C)** Quantification of ACh final responses, Kruskal-Wallis test significant between treatment groups, Mann-Whitney test $^{*}p<0.05$ Veh/Agonist vs Antagonist/Agonist. Data presented as mean ± S.E.M. N=4 rats, n=3-7 airways/rat.

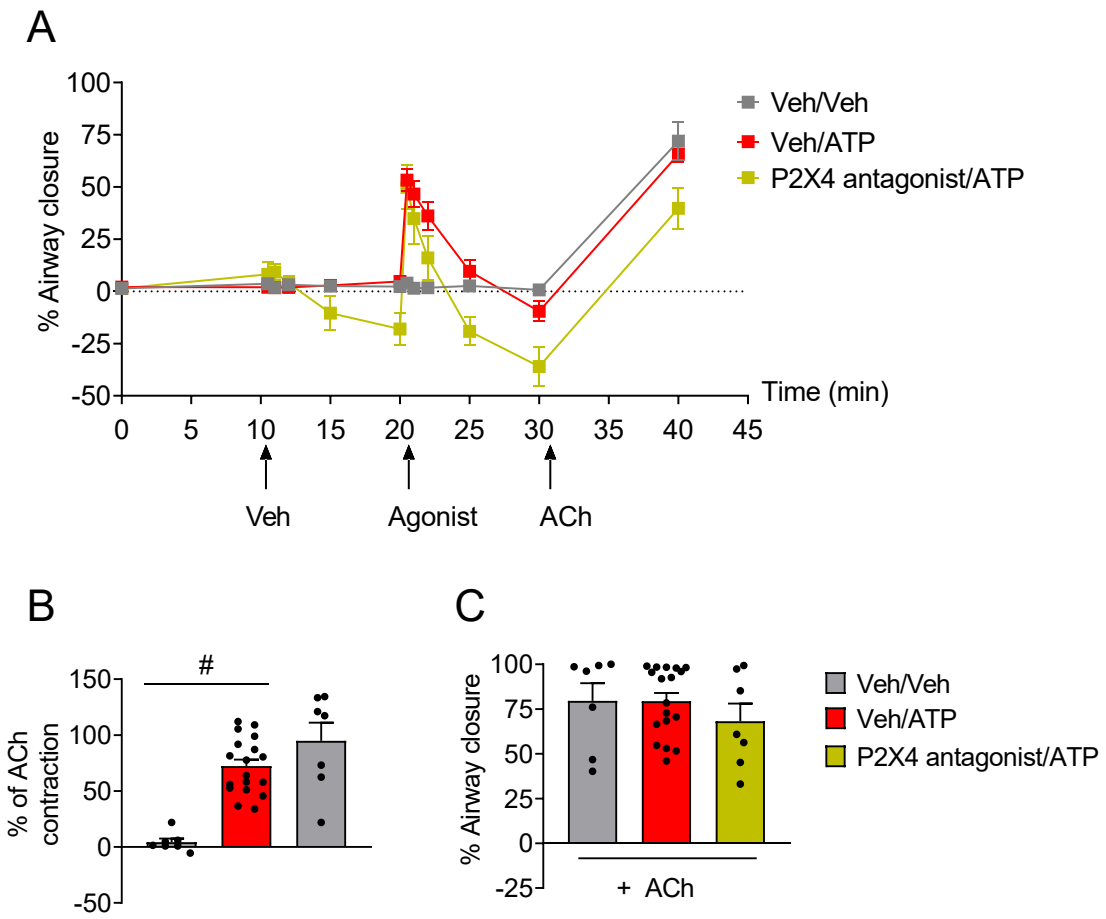


Figure 5-17 Effect of the P2X4 antagonist on ATP-induced small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or P2X4 antagonist 5-BDBD (10 μM) for 10 min and challenged with the vehicle (1% H_2O) or ATP (1 mM) for 10 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. **(A)** Trace of small airways contraction over time. **(B)** Quantification of maximum contraction normalised to ACh response, Mann-Whitney test $^{\#}p<0.05$ Veh/Veh vs Veh/agonist, non-significant Veh/Agonist vs Antagonist/Agonist. **(C)** Quantification of ACh final responses, Kruskal-Wallis non-significant. Data presented as mean ± S.E.M. N=3 rats, n=3-7 airways/rat.

5.5.10 Effect of mast cell mediator antagonists on TRPV4-induced Penh in naïve rats

These results suggested that TRPV4 activation induced small airways contraction in naïve rats via the release of ATP activating P2X4 receptors. In human and guinea pigs, TRPV4 was reported to provoke the contraction of large airways by inducing the release of ATP activating P2X4 receptors on mast cells and provoking the release of CysLTs (McAlexander et al. 2014; Bonvini et al. 2020) as schematised in (**Figure 5-19**).

CysLTs are one of the most potent bronchoconstrictors of human airways and are abundantly produced by human lung mast cells, along with histamine. In contrast, rodent airway smooth muscle appears less responsive to CysLTs whereas it contracts to 5-HT which is stored abundantly in rodent lung mast cells (Kushnir-Sukhov et al. 2007; Lulich and Paterson 1980; T. R. Martin et al. 1988; Muccitelli et al. 1987; Canning and Chou 2008; MacGlashan et al. 1982; Held, Martin, and Uhlig 1999; Seehase et al. 2011). Therefore, to account for potential species differences, a combination of antagonists of the main bronchoconstrictors 5-HT, histamine and LTD_4 derived from mast cells was tested against TRPV4-induced Penh in conscious naïve rats, namely methysergide (5-HTR antagonist), mepyramine (H1R antagonist) and montelukast (CysLT1R antagonist), to study if a similar mechanism was involved in rat small airways contraction.

Aerosolising the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) increased Penh AUC compared to the vehicle (1% EtOH) (689.7 ± 102.3 vs 4202 ± 893.5 Penh AUC). The combination of 5-HTR, H1R and CysLT1R antagonists methysergide (10 mg/kg, i.p.), mepyramine (10 mg/kg, i.p.) and montelukast (30 mg/kg, p.o.) did not prevent this increase compared to their vehicle (saline, i.p. and methylcellulose 0.5% Tween80 0.2%, p.o.) (4202 ± 893.5 vs 7723 ± 1545 Penh AUC) (**Figure 5-18**).

As a positive control, 5-HT (8 mg/ml) was aerosolised at the end of the experiment. The combination of methysergide, mepyramine and montelukast did not significantly reduce 5-HT induced Penh compared to their vehicle (**Figure 5-18**). This could be due to the confounding factor that animals were previously challenged with the TRPV4 agonist GSK1016790A.

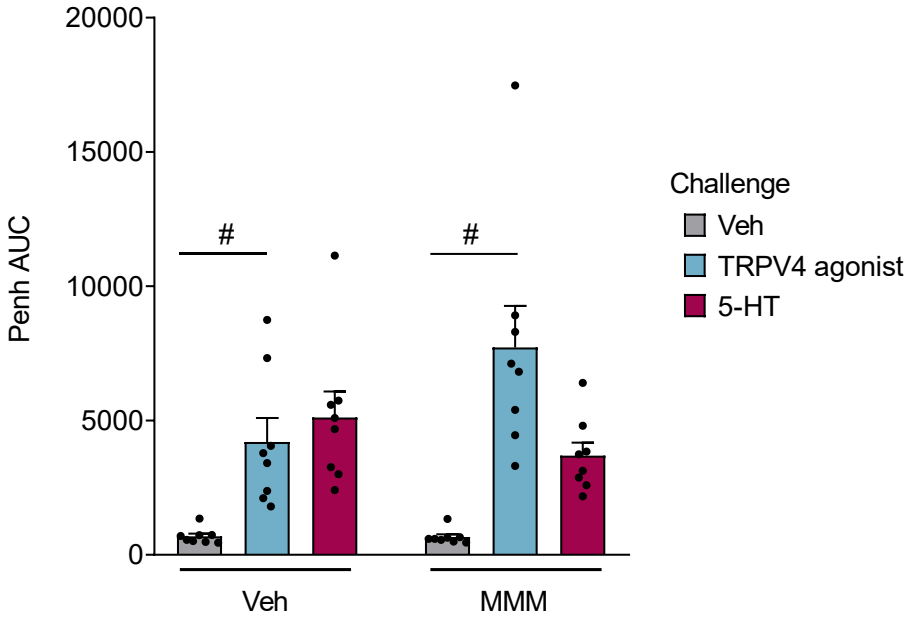


Figure 5-18 Effect of methysergide, mepyramine and montelukast on TRPV4-induced Penh

Conscious naïve rats were dosed with the combination (MMM) of 5-HTR antagonist methysergide (10 mg/kg, i.p.), H1R antagonist mepyramine (10 mg/kg, i.p.) and CysLTR1 antagonist montelukast (30 mg/kg, p.o.) or their vehicle (saline, i.p. and methylcellulose 0.5% Tween80 0.2%, p.o.) 30 min before challenge with the vehicle (1% EtOH) followed by the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) and by 5-HT (8 mg/ml) aerosolised for 10 min and Penh recorded for 20 min using FWBP. The graph represents the quantification of Penh AUC, N=8/group. Data presented as mean ± S.E.M. Wilcoxon test $^{\#}p<0.05$ agonist vs paired vehicle challenge; Mann-Whitney test non-significant between antagonist and vehicle treatments.

5.6 Discussion

Chapter 3 demonstrated that TRPV4 activation depolarised rat vagal nerves via the ATP-P2X3 axis and **Chapter 4** that TRPV4 and P2X3 antagonists significantly inhibited the LAR, supporting the hypothesis of the TRPV4-P2X3 axis driving a nerve reflex leading to the LAR. However, finding that TRPV4 and P2X3 antagonists separately reduce the LAR does not ascertain whether TRPV4 and P2X3 receptors are part of the same pathway in the disease model, especially considering the ubiquitous nature and multiple sources of ATP available during inflammation. More importantly, it does not prove with certainty that activation of TRPV4-P2X3 is involved in the LAR by triggering a cholinergic reflex. To address these uncertainties, one possible approach consisted of testing whether challenging naïve rats with the TRPV4 agonist could mimic the bronchospasm seen in the LAR, and if it was mediated by P2X3 receptors and a cholinergic reflex.

After determining a submaximal concentration in a concentration-response study, aerosolising a single dose of the TRPV4 agonist GSK1016790A (10 µg/ml or 15.25 µM) elicited a small magnitude and short-lasting increase in Penh in conscious naïve rats, blocked by the TRPV4 antagonist GSK2193874. Surprisingly, neither the P2X3-P2X2/3 antagonist AF-353 nor the LAMA tiotropium blocked this Penh response. This refuted the hypothesis that TRPV4 induced bronchospasm in naïve rats by activating P2X3-P2X2/3 receptors on afferent nerves and triggering efferent cholinergic nerves to release ACh.

These results were unexpected as TRPV4 activation elicited cough, another airway reflex, in guinea pigs using the same TRPV4 agonist at a similar concentration (30 µg/ml) and the effect was blocked by the same P2X3-P2X2/3 antagonist. In addition, nebulising this TRPV4 agonist provoked a sustained airway sensory Aδ-fibre firing in the anaesthetised rat (**3.5.4**), the same way it was shown to fire Aδ-fibres in guinea pigs (Bonvini et al. 2016). This discrepancy could be due to inter-species differences, with guinea pigs known to exhibit airway reactivity to nonspecific irritants and a cough reflex, whereas studies have failed to demonstrate the existence of a cough reflex in rodents (Canning and Chou 2008; Stevenson and Belvisi 2008; Zosky and Sly 2007).

Instead, these Penh findings suggested that TRPV4 activation provoked local bronchospasm in naïve rats independently from a nerve reflex. This idea is not itself surprising given the wide expression of TRPV4 in the airways (White et al. 2016) and the fact that TRPV4 was shown to contract human and guinea pig large airways. However, this was unexpected in the rat species as TRPV4 agonists were shown to contract isolated human bronchi and guinea pig trachea via the release of CysLTs (McAlexander et al. 2014; Bonvini et al. 2020) as

schematised in (**Figure 5-19**) and rodent airway smooth muscle is less sensitive to CysLTs (Held, Martin, and Uhlig 1999; T. R. Martin et al. 1988; Lulich and Paterson 1980; Seehase et al. 2011; Canning and Chou 2008). Accordingly, TRPV4 agonists did not provoke the contraction of rat or murine isolated tracheas (McAlexander et al. 2014; J. Zhang et al. 2019).

Before drawing the unexpected conclusion that TRPV4 provoked local bronchospasm in rats, it was important to confirm that this increase in Penh was synonym of bronchospasm. As previously explained in **2.3.2.2**, the Penh parameter can also be altered upon nose obstruction or modulation of the respiratory rhythm (Lomask 2006; Nakaya et al. 2006). Since the TRPV4 agonist GSK1016790A has been shown to alter respiratory frequency in rats and mice (Q. D. Gu et al. 2016; J. Zhang et al. 2019), it was especially necessary to confirm the effect of TRPV4 using a more direct measure of bronchospasm. This was assessed in the same single fibre preparation than used in **3.5.4.**: a naïve rat was anaesthetised, paralysed and intratracheal pressure invasively recorded via a pressure transducer. Both vagi were cut at their central end, preventing reflex bronchospasm. In these conditions, aerosolising the TRPV4 agonist directly into the tracheal cannula and thereby bypassing upper airways resulted in an immediate and short-lasting rise in intratracheal pressure. This preliminary finding seemed to confirm that TRPV4 activation provoked local bronchospasm independently from a central reflex. However, an important limit of this work was that only N=1 rat was used, due to the labour-intensive nature of the preparation and limited availability of high-skilled experts performing this technique as explained in **3.6**.

To confirm this preliminary finding, further experiments were conducted *in vitro* on airways isolated from naïve rats. This allowed to directly study the contraction of airways without influences from mucus secretion, oedema, nerve reflexes or pharmacokinetics considerations. Replicating experiments from the literature (McAlexander et al. 2014), the TRPV4 agonist GSK1016790A did not provoke the contraction of tracheal nor main bronchi segments isolated from naïve rats.

As the TRPV4 agonist increased Penh *in vivo* but did not contract rat isolated tracheas, it was hypothesised that TRPV4-induced bronchospasm was engaging intrapulmonary airways. To study this hypothesis, a preparation of rat PCLS was implemented. Airways selected for the experiments exhibited a diameter between 37 and 734 µm, with an average of 193 µm, corresponding to airways located below the 8th generation and therefore considered as small airways (Yeh, Schum, and Duggan 1979). As hypothesised, the TRPV4 agonist GSK1016790A elicited rat small airways contraction and this was inhibited by the TRPV4 antagonist GSK2193874. Although the TRPV4 antagonist seemed to induce baseline relaxation, it did not influence ACh contraction at the end of the experiment, perhaps

suggesting constitutive activity of the TRPV4 channel rather than a non-specific effect on the preparation. Overall, these data implied for the first time that TRPV4 activation provoked small airways contraction in naïve rats.

As previously outlined, studies from the laboratory have demonstrated that contraction of human bronchi induced by the TRPV4 agonist GSK1016790A was reduced by the P2X4 antagonist 5-BDBD and CysLT1R antagonist montelukast. In culture, the TRPV4 agonist induced the release of ATP from isolated human airway smooth muscle cells and only provoked their contraction when co-cultured with human lung mast cells (Bonvini et al. 2020). Thus, it is believed that activation of TRPV4 on airway smooth muscle cells induces the release of ATP that activates P2X4 receptors on mast cells and provokes the secretion of CysLTs that contract the airway smooth muscle (**Figure 5-19**). Studies were performed to decipher whether TRPV4-mediated rat small airways contraction involved a similar mechanism.

In PCLS from naïve rats, the TRPV4 agonist GSK1016790A-induced small airways contraction was inhibited by the Panx1 blocker probenecid, suggesting that ATP release through Panx1 channels could also mediate this effect, as it seems to be the case for many of TRPV4 actions (Belvisi and Birrell 2017). As a positive control, ATP also provoked rat small airways contraction, although with a sharp and transient peak as reported in the literature (Mounkaäila, Marthan, and Roux 2005; Bergner and Sanderson 2002b). The P2X3-P2X2/3 antagonist AF-353 had no effect on TRPV4 agonist-induced contraction, corroborating previous Penh findings. In contrast, the contraction was reduced by the P2X4 antagonist 5-BDBD, suggesting that TRPV4 activation contracted naïve rat small airways via the release of ATP activating P2X4 receptors, similar to demonstrated in human bronchi (Bonvini et al. 2020).

Conflicting with this hypothesis, the P2X4 antagonist 5-BDBD did not reduce the magnitude of ATP contraction. However, incubating PCLS with exogenous ATP at the concentration of 1 mM may not exert the same effects than ATP released *in situ* upon TRPV4 activation. One compelling piece of evidence is that ATP contracts rat trachea (Mounkaäila, Marthan, and Roux 2005) while the TRPV4 agonist does not (McAlexander et al. 2014). ATP can activate a wide range of P2X receptors and is rapidly metabolised by ectonucleotidases (ATPases) into ADP, AMP and adenosine which can in turn activate a variety of P2Y and adenosine receptors (Burnstock 2018). Depending on its concentration, ATP preferentially activates different P2X receptors (North 2002) and its effect presumably depends on where it is released and which receptors are locally expressed. Perhaps due to these reasons and to the poor availability and selectivity of P2X and P2Y antagonists, a very sparse and conflicting literature exists on the

contraction elicited by ATP. Several mechanisms have been cited, with among them the activation of P2Y receptors, either with an epithelial-dependent prostanoid release or an epithelial-independent effect, or the activation of P2X receptors generating a Na^+ influx and the opening of L-type calcium channels (Mounkaäila, Marthan, and Roux 2005; Flores-Soto et al. 2011; Gui et al. 2011; Bergner and Sanderson 2002b). P2X1 and P2X4 receptors have been cited as possible subtypes involved based on expression data and non-selective compounds (Nagaoka et al. 2009; Gui et al. 2011).

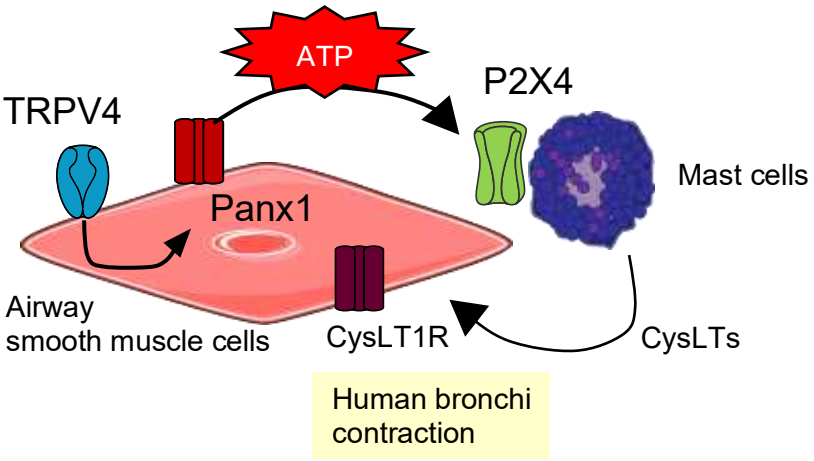


Figure 5-19 Mechanism of TRPV4-P2X4 induced contraction of human bronchi

Schematic of the hypothetic mechanism by which TRPV4 activation provokes human bronchi contraction demonstrated in (Bonvini et al. 2020). Activation of TRPV4 channel expressed on human airway smooth muscle cells provokes the release of ATP through Panx1 channels. The released ATP activates P2X4 receptors on human lung mast cells and triggers the release of CysLTs such as LTD_4 which bind to CysLTR1 receptors on airway smooth muscle cells and provoke contraction.

This lack of selective compounds also limited the studies in this thesis. Probenecid is a non-selective Panx1 blocker with a pIC_{50} of ~ 3.8 against Panx1 channels (Silverman, Locovei, and Dahl 2008) and while 5-BDBD preferentially inhibits rat P2X4 receptors, it was also shown to inhibit rat P2X1 and P2X3 receptors when used at 10 µM in HEK293 cells (Coddou et al. 2019). The contribution of P2X3-P2X2/3 receptors in TRPV4-induced contraction was however ruled out using AF-353 that was shown to be selective for P2X3 and P2X2/3 at the concentration used (Gever et al. 2010). In human bronchi, TRPV4-induced contraction was not reduced in presence of the P2X1 antagonist Ip_5I (10 µM) (Bonvini et al. 2020), yet it was not possible to test this antagonist in rat PCLS as Ip_5I was no longer commercially available.

Another limit of this work was the observation that both Panx1 blocker probenecid and P2X4 antagonist 5-BDBD seemed to relax small airways at baseline, an effect which could skew the

results. This may indicate that the ATP-P2X4 axis is constitutively active in the preparation but could also reveal an unspecific and/or toxic effect, especially for 5-BDBD which reduced ACh contraction. Due to potential off-target effects and lack of solubility of probenecid and 5-BDBD compounds, these antagonists were not tested *in vivo* against TRPV4-induced Penh.

Parallel work conducted during this thesis showed that mast cells extracted from Brown Norway rat lungs express *P2x4* mRNA and degranulate to ATP stimulation but not to the TRPV4 agonist GSK1016790A (Flajolet et al. 2019). Given these results, it was plausible to consider that activation of the TRPV4-P2X4 axis provokes the contraction of rat small airways by inducing mast cell mediators release as for human bronchi. Knowing that rat mast cells predominantly store 5-HT rather than CysLTs and that rat airway smooth muscle seems less responsive to CysLTs compared to human (Kushnir-Sukhov et al. 2007; Lulich and Paterson 1980; T. R. Martin et al. 1988; Muccitelli et al. 1987; Canning and Chou 2008; MacGlashan et al. 1982; Held, Martin, and Uhlig 1999; Seehase et al. 2011), a combination of antagonists targeting the main mast cells-derived bronchoconstrictors 5-HT, histamine and CysLTs (LTC_4, LTD_4) was used *in vivo*. The combination of these antagonists did not block the Penh increase caused by the TRPV4 agonist, suggesting that it is unlikely that the TRPV4-P2X4 axis causes rat small airways contraction via the release of mast cell mediators. In fact, these results were expected as rat mast cells density decreases alongside the airway tree contrary to human (Miller and Pemberton 2002; Bachelet, Bernaudin, and Fleury-Feith 1988) and rat tracheas do not contract to TRPV4 stimulation.

Therefore, how the TRPV4-ATP-P2X4 axis contracts rat small airways remains unknown. It is unlikely that TRPV4 triggers small airways contraction in an autocrine manner by activating P2X4 receptors on airway smooth muscle cells, as rat tracheal airway smooth muscle cells were found to express *Trpv4* and *P2x4* (in-house data and (Gui et al. 2011)) but rat tracheas do not contract to TRPV4 stimulation, although it is possible that airway smooth muscle cells present in small airways are phenotypically different from that located in the trachea.

It is intriguing that a calcium channel such as TRPV4 would require a secondary mediator to cause contraction while its activation directly elicits calcium influx in airway smooth muscle cells (Jia et al. 2004; Bonvini et al. 2020). However, studies have shown that calcium influx does not necessarily translate into contraction and can even provoke relaxation (Sanderson et al. 2008). Coincidentally, pre-contracted mouse tracheas relaxed to TRPV4 agonist GSK1016790A stimulation, an effect possibly mediated by activation of the IP_3 pathway (J. Zhang et al. 2019). In fact, this indirect mechanism of action might help to explain differences observed between rat trachea and small airways. Indeed, ATP has been shown to differentially contract rat extrapulmonary and intrapulmonary airways, with a higher amplitude of

intrapulmonary contraction and different sensitivity to purinergic antagonists according to the airway location (Mounkaäila, Marthan, and Roux 2005). It may be that the TRPV4-ATP axis activates different types of receptors and/or types of cells along the airway tree, with the final effect depending on the summation of contractile and relaxing pathways.

Overall, these findings suggest that TRPV4 activation contracts small airways in the naïve rat. This may have uncovered a novel mechanism by which TRPV4 provokes bronchospasm independently from CysLTs release. The relevance of these findings would need to be studied in human PCLS, as caution is needed when extrapolating rat small airways findings to human. Indeed, rodents exhibit a different branching pattern with fewer airway generations and different cell composition than human airways (Mercer et al. 1994; Meyerholz et al. 2018; J. H. T. Bates and Irvin 2003; Phalen and Oldham 1983). Furthermore, contraction to ATP seems to be species-dependent (Mounkaäila, Marthan, and Roux 2005; Flores-Soto et al. 2011).

As for the relevance to the LAR, small airways disease is increasingly viewed as an important pathological process in asthma (Postma et al. 2019) and small airways obstruction has been involved in the LAR in asthmatics (Stenberg et al. 2017; van der Wiel et al. 2013). It is possible that activation of the TRPV4-P2X4 axis contributes to the bronchospasm seen in the LAR, either in bronchi via a CysLTs-driven mechanism in human and possibly via contracting small airways, but it is unknown whether this would be the predominant contribution of TRPV4 in the LAR. As previously explained, due to potential off-target effects and poor solubility of the P2X4 antagonist 5-BDBD, it was not possible to test the involvement of P2X4 receptors in the LAR in the rat OVA model.

Most importantly, results from this chapter failed to support the hypothesis that TRPV4-P2X3 activation elicits a cholinergic reflex bronchospasm in naïve rats. However, this may be the case in naïve rats but not necessarily in the context of allergic airway inflammation and the LAR. Parallels have been drawn between inflammation-induced hyperalgesia and the airway hyperresponsiveness seen in airway disease. In the context of inflammatory pain, a stimulus can provoke a heightened reflex response compared to the normal state, a phenomenon named hyperalgesia, and this can be paralleled to the excessive bronchospasm or “hyperreflexia” seen in allergic asthma (Adcock 1999). In the somatosensory and visceral systems, mechanical pressure has been shown to stimulate nerve afferents and to induce reflex responses such as a withdrawal reflex of the paw or visceromotor responses. In the context of inflammation, this reflex response is heightened (Alessandri-Haber et al. 2003; 2006; Sipe et al. 2008). Interestingly, TRPV4 blockade reduced these efferent responses when the mechanical stimulus was applied in presence of inflammatory mediators, such as PGE_2, 5-HT and a PAR2 agonist, but not at baseline, while it reduced afferent nerve firing both

at baseline and during inflammation. Authors concluded that TRPV4 is involved in mechanical-induced hyperalgesia in the inflammatory state, but does not contribute to mechanical nociception in normal tissues (Alessandri-Haber et al. 2003; 2006; Sipe et al. 2008). A similar hypothesis could be drawn in the airways, that a TRPV4-P2X3 induced afferent nerve signal is not efficient to provoke an efferent response in the naïve state but could do so in allergic airway disease.

Accordingly, the concept that an afferent nerve signal can elicit an efferent response in the inflammatory but not naïve state has gained attention in asthma. Allergen challenge is associated with an increased cough reflex sensitivity to capsaicin and increased spontaneous coughing in atopic asthmatics (Satia et al. 2019), illustrating an enhancement of airway reflexes in presence of allergic airway inflammation. Several mechanisms have been highlighted as potential causes of the hyperreflexia seen in asthma (Undem and Taylor-Clark 2014; Undem and Carr 2002; Mazzone and Undem 2016), which could help to draw hypotheses on how a TRPV4-P2X3 nerve signal could trigger a reflex in the LAR but not in the naïve state, as illustrated in (**Figure 5-20**):

- Firstly, inflammation has been shown to enhance airway sensory nerve activation, notably with inflammatory mediators increasing afferent nerves excitability (Undem and Taylor-Clark 2014). In asthma, the signal induced by TRPV4 could be enhanced by the downregulation of the main enzymes catabolising ATP (CD39 and CD73 ectonucleotidases) which has been observed in asthmatic patients (Antonioli et al. 2019; L. L. Wang et al. 2013; 2014). Airway inflammation may also alter or create new cellular interactions between $TRPV4^+$ cells and airway sensory nerves (L. A. Laitinen et al. 1985) and upregulate P2X3 receptors on airway sensory nerves (Barrios et al. 2017).

- Secondly, inflammation has been shown to increase synaptic efficacy at the CNS level. As such, studies conducted in guinea pigs showed that stimulation of airway sensory C-fibres increased the cholinergic bronchospasm provoked by activation of Aδ-fibres. This synergy between Aδ- and C-fibres was blocked by administering tachykinin antagonists centrally, suggesting that C-fibres activation could release tachykinins in the CNS and enhance Aδ-fibres output on cholinergic centres (Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002b). This finding is especially relevant for this thesis, as TRPV4 is thought to activate Aδ-fibres and many inflammatory mediators elevated after allergen challenge are known activators of C-fibres, such as bradykinin or the eicosanoid PGD_2 (Christiansen et al. 1992; M. C. Liu et al. 1991; O'Sullivan et al. 1998; Fox et al. 1993; Maher et al. 2015).

- Thirdly, inflammation has been shown to increase efferent parasympathetic signalling (Undem and Taylor-Clark 2014). Among studied mechanisms, several reports have highlighted a downregulation of muscarinic M2 receptors in asthmatic patients and after allergen challenge in guinea pigs. M2 are inhibitory receptors situated on postganglionic cholinergic nerve terminals and their activation limits ACh release through a negative feedback loop (Minette et al. 1989; R. E. ten Berge et al. 1996; Fryer and Wills-Karp 1991). Therefore, downregulation of M2 receptors could enhance ACh release for a same afferent signal.

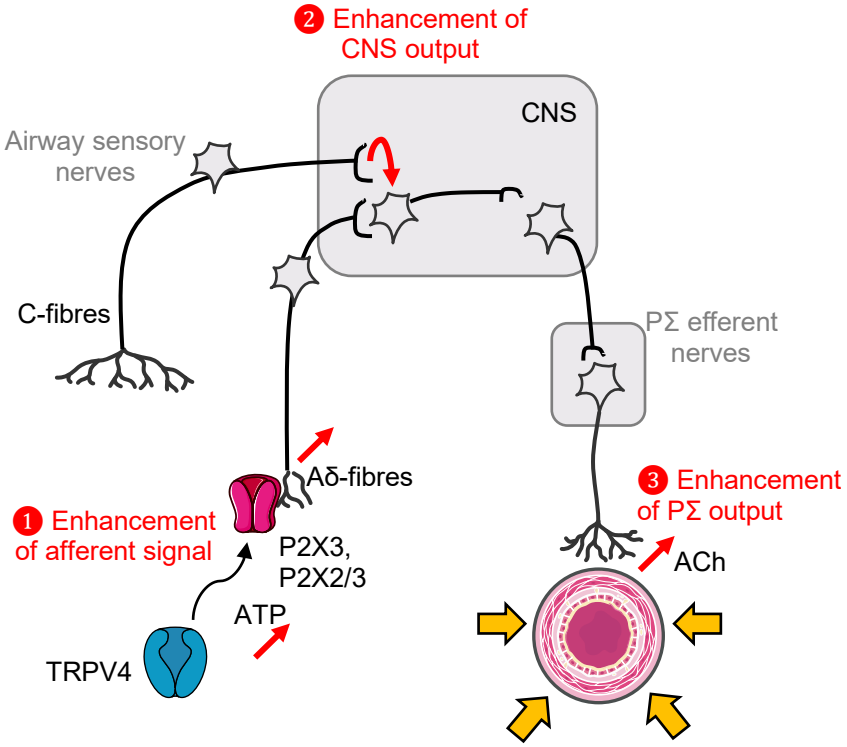


Figure 5-20 Hypothetical mechanisms of how inflammation could modulate the TRPV4-P2X3 nerve axis in allergic airway disease

Hypothetical mechanisms that could explain how the TRPV4-P2X3 axis could trigger a cholinergic reflex bronchospasm in the context of allergic airway inflammation but not in naïve rats. (1) Inflammatory mediators could enhance the airway sensory Aδ-fibres signal elicited by the TRPV4-P2X3 axis. (2) Inflammatory mediators could enhance CNS synaptic efficacy by activating airway sensory C-fibres and increasing the output of Aδ-fibres on cholinergic centres. (3) Inflammatory mediators could enhance the release of ACh from parasympathetic cholinergic nerves, for instance by inhibiting autoinhibitory M2 receptors. Schematics based upon concepts presented in the extensive review by (Undem and Taylor-Clark 2014). CNS: Central Nervous System. PΣ: Parasympathetic nerves.

There is also a possibility that TRPV4 receptors are involved in enhancing the efferent arm of the reflex rather than being involved in the afferent arm, although this seems unlikely given the sustained airway sensory nerve firing elicited by the TRPV4 agonist in naïve rat and guinea pigs (Bonvini et al. 2016). In this chapter, neither ACh-induced Penh response nor ACh-induced small airways contraction in PCLS was blocked by the TRPV4 antagonist GSK2193874, suggesting that TRPV4 is not involved downstream of ACh release. However, it was not studied whether TRPV4 activation could facilitate ACh release from cholinergic nerve terminals, which could be possible as rat parasympathetic tracheal ganglia express P2X2 receptors (B. Ma et al. 2005).

To conclude this chapter, the TRPV4 agonist seemed to elicit bronchospasm in the naïve rat by inducing local small airways contraction via P2X4 receptors, independently from a P2X3-mediated cholinergic reflex. It can be hypothesised that activation of the TRPV4-P2X3 nerve axis does not elicit a reflex in the naïve rat but may do so in presence of allergic airway inflammation. Studying possible mechanisms triggering the TRPV4-P2X3 axis could provide insights as to whether this axis is causing a cholinergic reflex in the LAR.

6 Investigation of PAR2 receptors as TRPV4 activators

6.1 Introduction

Results from **Chapters 3 and 4** suggested that TRPV4 and P2X3-P2X2/3 receptors were involved in driving the LAR in the rat OVA model, with allergen challenge resulting in the activation of TRPV4, leading to the release of ATP activating P2X3-P2X2/3 receptors on airway sensory nerves and a subsequent cholinergic reflex bronchospasm. According to results from **Chapter 5**, it was hypothesised that activation of this TRPV4-P2X3 afferent nerve signal may elicit reflex bronchospasm in presence of airway inflammation but not in the naïve state. However, it remains unknown how allergen challenge would result in the activation of the TRPV4-P2X3 axis, that would trigger a delayed and prolonged bronchospasm characteristic of the LAR. Therefore, the final aim of this thesis was to investigate potential activators of the TRPV4-P2X3 axis in the LAR.

Many stimuli of different modalities have been associated with TRPV4 opening and yet it is still unclear which precise endogenous ligands stimulate this channel (White et al. 2016). Protease-activated receptors 2 (PAR2) have been increasingly linked to TRPV4 signalling (Cenac et al. 2015; Grant et al. 2007; Poole et al. 2013; Zhao et al. 2015; Sipe et al. 2008; Zhao et al. 2014; Grace et al. 2014). PAR2 receptors are GPCRs activated by proteolytic cleavage of their N-termini by proteases such as tryptase, revealing a ligand sequence on the receptor itself that binds back to an extracellular loop and trigger G-protein signalling (Adams et al. 2011). Relevant to this thesis, PAR2 receptors have been shown to activate and/or sensitise TRPV4 channels, leading to a signal in sensory neurons in the somatosensory and visceral systems (Cenac et al. 2015; Grant et al. 2007; Poole et al. 2013; Zhao et al. 2015; Sipe et al. 2008). More importantly, PAR2-mediated activation of TRPV4 seems to provoke mechanical hyperalgesia by causing afferent nerves firing and efferent responses (Cenac et al. 2015; Grant et al. 2007; Poole et al. 2013; Zhao et al. 2015; Sipe et al. 2008). These findings highlighted PAR2 as a promising candidate to trigger a TRPV4-mediated reflex in presence of inflammation.

In addition, many studies have reported a role for PAR2 in allergic airway disease. PAR2 receptors are widely expressed in the airways (Cocks and Moffatt 2001) and upregulated in the airways of asthmatic patients (Knight et al. 2001; Allard et al. 2014; Aubier et al. 2016). Agonists of PAR2 receptors such as tryptase, tissue factor and kallikrein 14 were also found upregulated (Aubier et al. 2016). In mouse models, PAR2 blockade was associated with

diminished allergic airway inflammation and AHR in OVA, HDM and cockroach extract driven models (Schmidlin et al. 2002; Davidson et al. 2013; de Boer et al. 2014; Asaduzzaman et al. 2015, 2018). Although the involvement of PAR2 has never been studied in allergen-induced bronchospasm, allergen challenge is associated with mast cell degranulation and the release of the potent PAR2 agonist tryptase in the airways of asthmatic patients (S. E. Wenzel, Fowler, and Schwartz 1988; Sedgwick et al. 1991), with early studies showing that tryptase inhibitors reduced the LAR in human and allergic sheep models (Clark et al. 1995; Costanzo et al. 2003; Krishna et al. 2001). Therefore, it was hypothesised that PAR2 receptors could be promising candidates for activating a TRPV4-mediated nerve reflex in the LAR.

6.2 Hypothesis

Allergen challenge triggers the release of proteases such as tryptase from mast cells that activate PAR2 receptors and provoke a TRPV4-P2X3 afferent nerve signal causing reflex bronchospasm in the LAR (**Figure 6-1**).

6.3 Aims

- Study the expression of PAR2 agonists in the rat OVA model.
- Investigate the effect of PAR2 activation on airway sensory nerves *in vitro* and *in vivo* and if it is mediated via the TRPV4-P2X3 axis.
- Investigate if PAR2 activation can provoke bronchospasm in naïve rats and if it is mediated via a reflex or local airway smooth muscle contraction *in vivo* and *in vitro*.
- Investigate if PAR2 receptors are involved in the LAR using PAR2 antagonists in the rat OVA model.

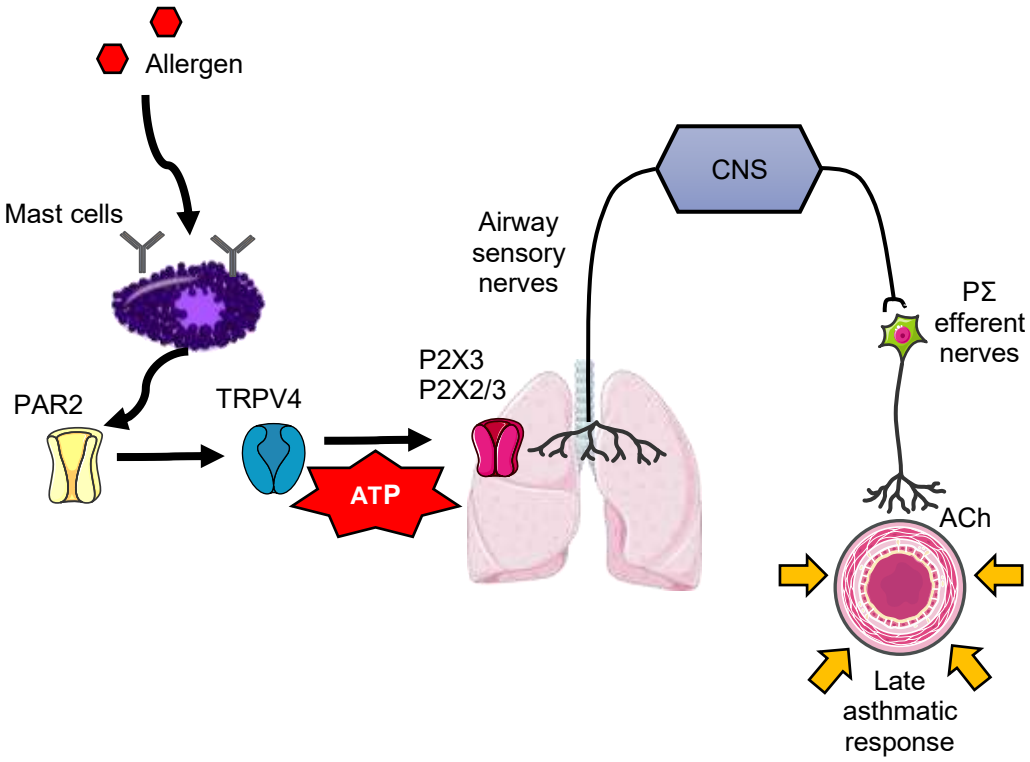


Figure 6-1 Hypothesis: PAR2 receptors activate the TRPV4-P2X3 axis in the LAR

It is hypothesised that allergen challenge triggers the release of proteases such as tryptase from mast cells that activate PAR2 receptors, leading to a TRPV4-P2X3 afferent nerve signal and a reflex bronchospasm driving the LAR. CNS: Central Nervous System. PΣ: Parasympathetic.

6.4 Methods

6.4.1 Time-course of tryptase-like activity in the rat OVA model

To assess the regulation of PAR2 agonists in the rat OVA model, tryptase-like activity was measured in the time-course experiment described in **4.4.1**. Male Brown Norway rats (200-250 g) were sensitised on days D0, D14, D21 with OVA (in Alum™ diluted 1:1 in saline). On D28, rats were challenged either with OVA or the vehicle SAL (saline) aerosolised for 30 min. After challenge, rats were euthanised with an overdose of pentobarbitone (200 mg/kg, i.p.) at 1, 2, 6 and 24 h post-challenge to harvest BAL samples as described in **2.3.3**, with N=6 rats/group per timepoint as represented in **4.4.1**.

Tryptase-like activity was measured by incubating BAL samples with the tryptase substrate tosyl-gly-pro-lys-*p*NA and measuring the product *p*NA which absorbs at 405 nm as described in **2.4.2**.

Data were expressed as the absorbance at 405 nm minus appropriate blanks representing levels of tryptase-like activity in the BAL samples. Non-parametric Mann Whitney tests compared absorbance levels between SAL and OVA challenge for each timepoint.

6.4.2 Effect of PAR2 agonists on isolated vagus depolarisation

The effect of PAR2 activation was tested on the depolarisation of vagal nerves using the isolated vagus preparation detailed in **2.2.1** generated from naive rats and *Par2*$^{-/-}$and *Par2*$^{+/+}$ mice.

6.4.2.1 Effect of PAR2 agonists on rat vagus depolarisation

Concentration-responses of a range of PAR2 agonists were conducted on vagal nerves isolated from naïve rats. Each segment was incubated with the vehicle (0.1% DMSO or 0.1% H_2O) followed by increasing concentrations of either: trypsin (30 – 1'000 U/ml or ~ 500 nM - 16 µM), 2f-LIGRLO-NH2 (1 – 1'000 nM), SLIGKV-NH2 (10 – 10'000 nM) or AC55541 (10 – 10'000 nM). Each drug concentration was applied for 2 min and washed for 10 min before the next application. Nerve viability was assessed at the beginning and end of experiment by incubating the nerve with the TRPV1 agonist capsaicin (1 µM).

6.4.2.2 Effect of the PAR2 agonist on vagal nerves from *Par2*$^{-/-}$ mice

The selectivity of the PAR2 agonist 2f-LIGRLO-NH2 was tested in vagal nerves isolated from naïve *Par2*$^{-/-}$ and littermate *Par2*$^{+/+}$ mice. Each segment was successively incubated with the

vehicle (0.1% DMSO), PAR2 agonist 2f-LIGRLO-NH2 (100 nM), TRPV4 agonist GSK1016790A (300 nM) and TRPV1 agonist capsaicin (1 µM). Each drug was applied for 2 min and washed for 10 min before the next application. The genotype of *Par2*$^{+/+}$ and *Par2*$^{-/-}$ mice was confirmed according to the protocol described in **2.5**.

6.4.2.3 Effect of PAR2, TRPV4 and P2X3-P2X2/3 antagonists on rat PAR2-mediated vagus depolarisation

Vagal nerves were isolated from naïve rats. Each segment was incubated with a submaximal concentration of the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) for 2 min. This was repeated after a wash to ensure reproducibility of the response. The nerve was subsequently incubated with the antagonist for 10 min, either the PAR2 antagonist P2pal-18S (10 µM), control Scrambled peptide (10 µM), TRPV4 antagonist GSK2193874 (10 µM), P2X3-P2X2/3 antagonist AF-353 (10 µM) or vehicle (0.1% DMSO). The PAR2 agonist was then reapplied for 2 min in presence of the antagonist. After washing, the nerve was re-incubated with the PAR2 agonist to establish viability and recovery of the response.

6.4.2.4 Selection of PAR2 antagonist P2pal-18S concentration for *in vitro* use

The PAR2 antagonist P2pal-18S was shown to be selective for PAR2 receptors (Sevigny et al. 2011). Because it was custom-made in a limited quantity, only a single concentration of the antagonist could be tested. The concentration of 10 µM was chosen as it blocked PAR2 signalling *in vitro* (Bagher et al. 2018; Lin et al. 2015) and corresponded to approximately 100 times its reported IC_{50} (0.14 – 0.2 µM) (Sevigny et al. 2011).

6.4.2.5 Vagus data analysis

Vagus data were expressed as mean ± S.E.M. depolarisation (mV) and percentage inhibition calculated for each antagonist. Statistical analysis was performed on the depolarisation (mV) data. Parametric paired t-tests compared the depolarisation elicited by the agonist in absence and presence of the antagonist. Unpaired t-tests compared the depolarisation elicited by the agonist in *Par2*$^{+/+}$ compared to *Par2*$^{-/-}$ mice. One-way ANOVA followed by Dunnett's multiple comparisons test compared the depolarisation elicited by increasing concentrations of an agonist versus the vehicle control.

6.4.3 Effect of a PAR2 agonist on airway sensory Aδ-fibre firing and intratracheal pressure in anaesthetised rats

The ability of PAR2 activation to provoke airway sensory Aδ-fibre firing and to elicit local bronchospasm independently from a nerve reflex was tested *in vivo* using the single fibre preparation in anaesthetised naïve rats. Both vagi were disconnected from their central end (bilateral cervical vagotomy) to avoid central reflexes. Airway sensory Aδ-fibre action potential firing was measured as detailed in **2.2.3**. Local bronchospasm was monitored by measuring intratracheal pressure as detailed in **2.6.2**.

6.4.3.1 Effect of the PAR2 agonist concentration-response

After identification of an airway sensory Aδ-fibre and establishing baseline activity, the vehicle (0.1% DMSO) followed by increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 (1 – 1'000 nM) were aerosolised via a tracheal cannula. Each concentration was aerosolised for 1 min and the effect on Aδ-fibre action potential firing and intratracheal pressure observed for 10 min before the next concentration was applied. At the end of the experiment, citric acid (0.3 M) was aerosolised for 1 min to ensure viability of the preparation.

6.4.3.2 Effect of PAR2 antagonist on PAR2-induced Aδ-fibre firing

After identification of an airway sensory Aδ-fibre and establishing baseline activity, the PAR2 agonist 2f-LIGRLO-NH2 (1 µM) was aerosolised for 1 min via a tracheal cannula and the effect on Aδ-fibre firing and intratracheal pressure observed. This was repeated after 10 min to ensure reproducibility of the response. The PAR2 antagonist P2pal-18S (10 mg/kg in 5% DMSO in saline, s.c.) was then administered subcutaneously. After 30 min, the PAR2 agonist 2f-LIGRLO-NH2 (1 µM) was aerosolised for 1 min. At the end of the experiment, citric acid (0.3 M) was aerosolised for 1 min to ensure viability of the preparation.

6.4.3.3 Selection of the PAR2 antagonist P2pal-18S dose for *in vivo* use

Due to the limited availability of the PAR2 antagonist P2pal-18S, only a single dose of the PAR2 antagonist could be tested. The dose of 10 mg/kg, s.c. was chosen as it was shown to elicit plasma levels of approximately 10 µM within the first 4 h in mice, with distribution found in the lungs (Shearer et al. 2016) and to inhibit PAR2 responses *in vivo* (Sevigny et al. 2011). The compound was dissolved in a high concentration of DMSO (5% in saline) due to its low solubility conferred by the palmitate residue (Lin et al. 2015).

6.4.3.4 Single fibre data analysis

Data were analysed as the frequency of action potential firing events of Aδ-fibres (impulses/sec) and changes in intratracheal pressure (cm H_2O) over time. Statistical analysis was not performed as only N=1 experiment was conducted for each study.

6.4.4 Effect of a PAR2 agonist on isolated trachea contraction

The ability of PAR2 to contract extrapulmonary airways was tested *in vitro* using organ bath preparations of tracheas isolated from naïve rats as described in **2.6.3**.

Tracheas were generated from naïve rats and incubated with ACh (1 mM) to determine maximal contraction. After several washes and return to baseline tension, tissues were incubated with a single concentration of the vehicle (0.1% DMSO) or PAR2 agonist 2f-LIGRLO-NH2 (10 – 1'000 nM). Changes in isometric tension (mg) were monitored using a force transducer to assess tissue contraction. After 40 min, tissues were washed and ACh (1 mM) was reapplied to ensure tissue viability was not affected by drug treatment. The experiment was conducted in presence of the COX inhibitor indomethacin (10 µM).

Data were expressed as the maximum tension (mg) elicited by a compound during the 40-min incubation. For statistical analysis, data were normalised to the maximal contraction elicited by ACh for each tissue segment and analysed using non-parametric Kruskal-Wallis test comparing the effect of different agonist concentrations with the vehicle control.

6.4.5 Effect of a PAR2 agonist on small airways contraction using PCLS

The ability of PAR2 to contract intrapulmonary airways was tested *in vitro* using PCLS generated from naïve rats as described in **2.6.4**.

On day 1 post-isolation, PCLS were incubated with ACh (1 mM) to select viable airways contracting more than 50% to ACh. Airways with a similar range of diameters and ACh responses were distributed among treatment groups. On day 2 post-isolation, pharmacological experiments were performed. Airway contraction was monitored using light microscopy.

Baseline images were taken of each identified airway. Slices were incubated with a single concentration of either the vehicle (0.1% DMSO) or PAR2 agonist 2f-LIGRLO-NH2 (1 - 1'000 nM) for 30 min. Images were taken every 5 min. Without washing, ACh (1 mM) was added for 10 min to assess airway viability and maximum contractility at the end of the experiment.

Contraction was plotted over time by measuring airway lumen area and calculating the percentage closure from baseline at each timepoint. To analyse the contraction elicited by an agonist, the percentage closure was calculated from the baseline area captured right before agonist incubation. For statistical analysis, the maximum percentage closure elicited during agonist incubation was normalised to the contraction elicited by final incubation with ACh (1 mM) and Mann-Whitney tests were used to compare between specified groups.

6.4.6 Effect of a PAR2 agonist on Penh in conscious naïve rats

The ability of PAR2 activation to induce reflex bronchospasm was tested in conscious naïve rats by recording Penh using FWBP as described in **2.6.1**.

Conscious naïve rats were placed in FWBP chambers. After establishing a 5-min baseline, rats were challenged with increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 (10 nM - 10 µM) or corresponding dilutions of the vehicle (DMSO in saline) as shown in (**Figure 6-2**). Each concentration was aerosolised for 10 min and Penh recorded for 20 min, and a 5-min recovery period was set between each challenge.

N	Challenge 1	Challenge 2	Challenge 3	Challenge 4	Challenge 5
6	Veh (0.00001%)	Veh (0.0001%)	Veh (0.001%)	Veh (0.01%)	Veh (0.1%)
6	Veh (0.00001%)	PAR2 agonist 10 nM	PAR2 agonist 100 nM	PAR2 agonist 1 µM	PAR2 agonist 10 µM

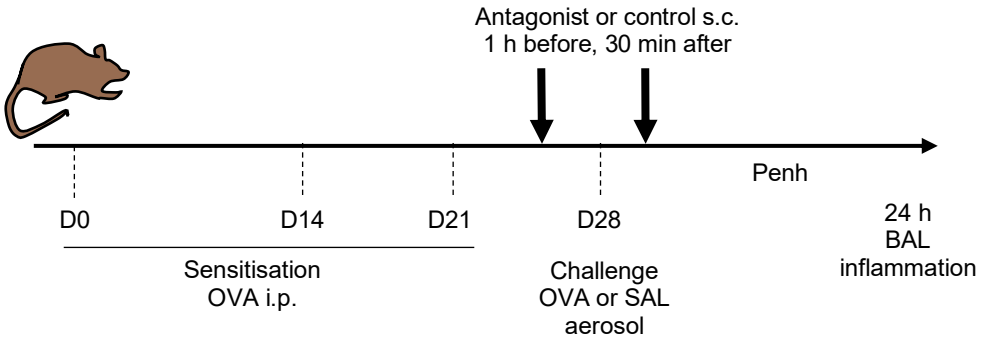
Figure 6-2 Protocol of PAR2 agonist concentration-response Penh study
Veh: DMSO in saline with the corresponding DMSO dilution (%)
PAR2 agonist: 2f-LIGRLO-NH2

Penh data were averaged every min and plotted over time to calculate Penh AUC. Statistical analysis was performed on Penh AUCs. Mann-Whitney tests were used to compare data between agonist and time-matched vehicle challenge.

6.4.7 PAR2 antagonists in the rat OVA model

6.4.7.1 PAR2 antagonist P2pal-18S

The PAR2 antagonist P2pal-18S was tested against the LAR in the rat OVA model. Male Brown Norway rats were sensitised on days D0, D14, D21 with OVA (Alum™ diluted 1:1 in saline) as described in **2.3.1**. On D28, rats were challenged with SAL or OVA aerosol for 30 min. One hour before and 30 min after challenge, rats were dosed with the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.), control Scrambled peptide (10 mg/kg, s.c.) or vehicle (5% DMSO in saline, s.c.) with N=8 rats/group (**Figure 6-3**). One hour after challenge, rats were placed in FWBP chambers to assess Penh from 1 to 6 h post-challenge as described in **2.3.2**. BAL cells were harvested 24 h post-challenge to perform inflammatory cells differential counts as described in **2.3.3**.



N	Sensitisation	Challenge	Treatment s.c.
8	OVA	SAL	Veh 5% DMSO
8	OVA	SAL	Scrambled
8	OVA	SAL	PAR2 antagonist
8	OVA	OVA	Veh 5% DMSO
8	OVA	OVA	Scrambled
8	OVA	OVA	PAR2 antagonist

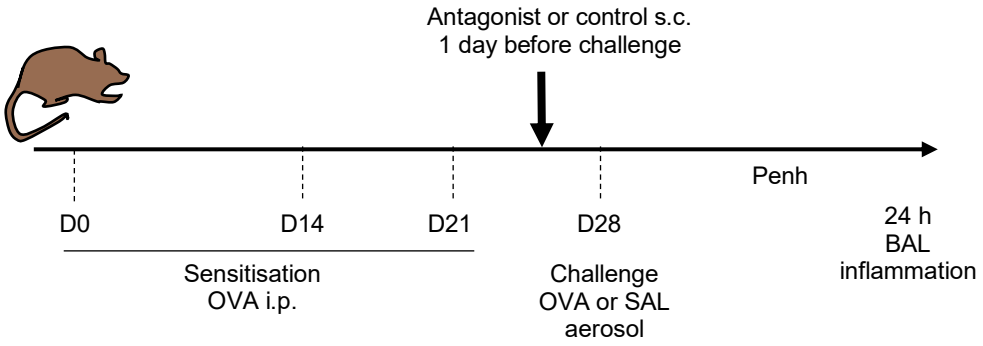
Figure 6-3 Protocol of PAR2 antagonist P2pal-18S study in the rat OVA model

6.4.7.2 Selection of PAR2 antagonist P2pal-18S dose for the rat OVA model

The dose of PAR2 antagonist was validated to block PAR2 agonist 2f-LIGRLO-NH2 induced Aδ-fibre firing in the anaesthetised rat. In mice, P2pal-18S exhibited a plasma elimination half-life of 3.1 h (Shearer et al. 2016). The compound was dosed 1 h before and 30 min after challenge to ensure sufficient exposure throughout the duration of the LAR and inflammatory cell recruitment.

6.4.7.3 Anti-PAR2 monoclonal antibody MEDI2344

The anti-PAR2 monoclonal antibody (mAb) MEDI2344 was tested against the LAR in the rat OVA model. Male Brown Norway rats were sensitised on days D0, D14, D21 with OVA (Alum™ diluted 1:1 in saline) as described in **2.3.1**. One day before challenge, rats were dosed with the anti-PAR2 monoclonal antibody MEDI2344 (10 mg/kg, s.c.), isotype IgG control (10 mg/kg, s.c.) or vehicle (25 mM Histidine, 205 mM sucrose in saline or "His-sucrose", s.c.) with N=8 rats/group (**Figure 6-4**). On D28, rats were challenged with SAL or OVA aerosol for 30 min., One hour after challenge, rats were placed in FWBP chambers to assess Penh from 1 to 6 h post-challenge as described in **2.3.2**. BAL cells were harvested 24 h post-challenge to perform inflammatory cells differential counts as described in **2.3.3**.



N	Sensitisation	Challenge	Treatment s.c.
8	OVA	SAL	Veh
8	OVA	SAL	Isotype IgG
8	OVA	SAL	PAR2 Ab
8	OVA	OVA	Veh
8	OVA	OVA	Isotype IgG
8	OVA	OVA	PAR2 mAb

Figure 6-4 Protocol of anti-PAR2 mAb MEDI2344 study in the rat OVA model

6.4.7.4 Selection of PAR2 monoclonal antibody MEDI2344 dose for the rat OVA model

The dose of anti-PAR2 monoclonal antibody MEDI2344 was shown to inhibit PAR2-induced pain responses in rats. Based on pharmacokinetic studies conducted in the rat, the antibody was dosed 1 day before challenge to ensure sufficient exposure throughout the duration of the LAR and inflammatory cell recruitment.

6.4.7.5 Penh data analysis

To analyse the LAR, average Penh was calculated every 10 min and plotted over time. Statistical analyses were performed on Penh AUCs data. Non-parametric Mann-Whitney tests compared data between specified groups.

To analyse airway inflammation, statistical analyses were performed on BAL inflammatory cell counts expressed as (10^3 cells/ml). Non-parametric Mann-Whitney tests compared data between specified groups.

6.5 Results

6.5.1 PAR2 agonists levels in the rat OVA model

The first aim of this chapter was to study whether PAR2 agonists were upregulated in the rat OVA model.

The regulation of PAR2 agonists was assessed in the rat OVA model in the same time-course experiment where ATP levels were evaluated (**4.5.1**). The level of tryptase-like proteases was assessed by measuring the cleavage of the substrate tosyl-gly-pro-lys-*p*NA. Tryptase-like activity was upregulated in the BAL of OVA-sensitised rats at 2, 6 and 24 h after OVA challenge compared to SAL challenge (**Figure 6-5**).

These results suggested an upregulation of PAR2 signalling in the rat OVA model, validating the use of this model to study the role of PAR2 receptors.

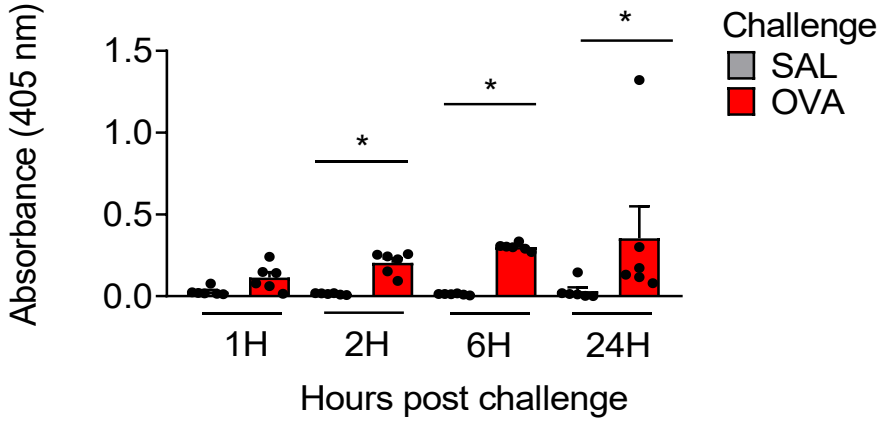


Figure 6-5 Tryptase-like activity in BAL samples in the rat OVA model

Rats were sensitised to OVA and challenged with OVA or SAL aerosol. BAL fluids were harvested 1, 2, 6 and 24 h post-challenge to assess tryptase-like activity levels by cleavage of the substrate tosyl-gly-pro-lys-*p*NA into *p*NA absorbing at 405 nm. Data is expressed as mean ± S.E.M., N=6 rats per group per timepoint. Mann-Whitney test *p<0.05 comparing between SAL and OVA challenge at the same timepoint.

6.5.2 Effect of PAR2 agonists on isolated rat vagus depolarisation

As it was hypothesised that PAR2 receptors could activate the TRPV4-P2X3 axis, leading to a nerve reflex leading the LAR, the second aim of this chapter was to test whether PAR2 activation can stimulate airway sensory nerves in naïve rats and if it is mediated via the TRPV4-P2X3 axis.

Since it was the first time that PAR2 was investigated in the laboratory, concentration-responses of several PAR2 agonists were conducted on the depolarisation of vagal nerves isolated from naïve rats, with the endogenous agonist trypsin and synthetic agonists 2f-LIGRLO-NH2, SLIGKV-NH2 and AC55541. The TRPV1 agonist capsaicin (1 μM) was applied at the end of each experiment to serve as a reference stimulus and to assess nerve viability.

All PAR2 agonists induced the depolarisation of rat vagal nerves compared to the vehicle (0.1% DMSO or 0.1% H_2O), with the following depolarisations at the highest tested concentrations: trypsin (1000 U/ml or ~16 μM): 0.20 ± 0.03 mV; 2f-LIGRLO-NH2 (1 μM): 0.09 ± 0.02 mV; SLIGKV-NH2 (10 μM): 0.07 ± 0.02 mV and AC55541 (10 μM): 0.05 ± 0.01 mV (**Figure 6-6**).

The most potent and selective PAR2 agonist was taken forward to pharmacology studies. The endogenous agonist trypsin was not selected as it has been reported to target PAR1 receptors (Heuberger and Schuepbach 2019). The synthetic agonists 2f-LIGRLO-NH2, SLIGKV-NH2 and AC55541 have been shown to be selective to PAR2 over PAR1 (Hollenberg et al. 1997; McGuire et al. 2004; Kawabata et al. 2004; Gardell et al. 2008), however 2f-LIGRLO-NH2 was preferred as the PAR2 agonist of choice as it seemed to be the most potent in eliciting vagus depolarisation. The concentration of 100 nM 2f-LIGRLO-NH2 that elicited submaximal vagus depolarisation amongst tested concentrations was taken forward to antagonist studies (**Figure 6-6**).

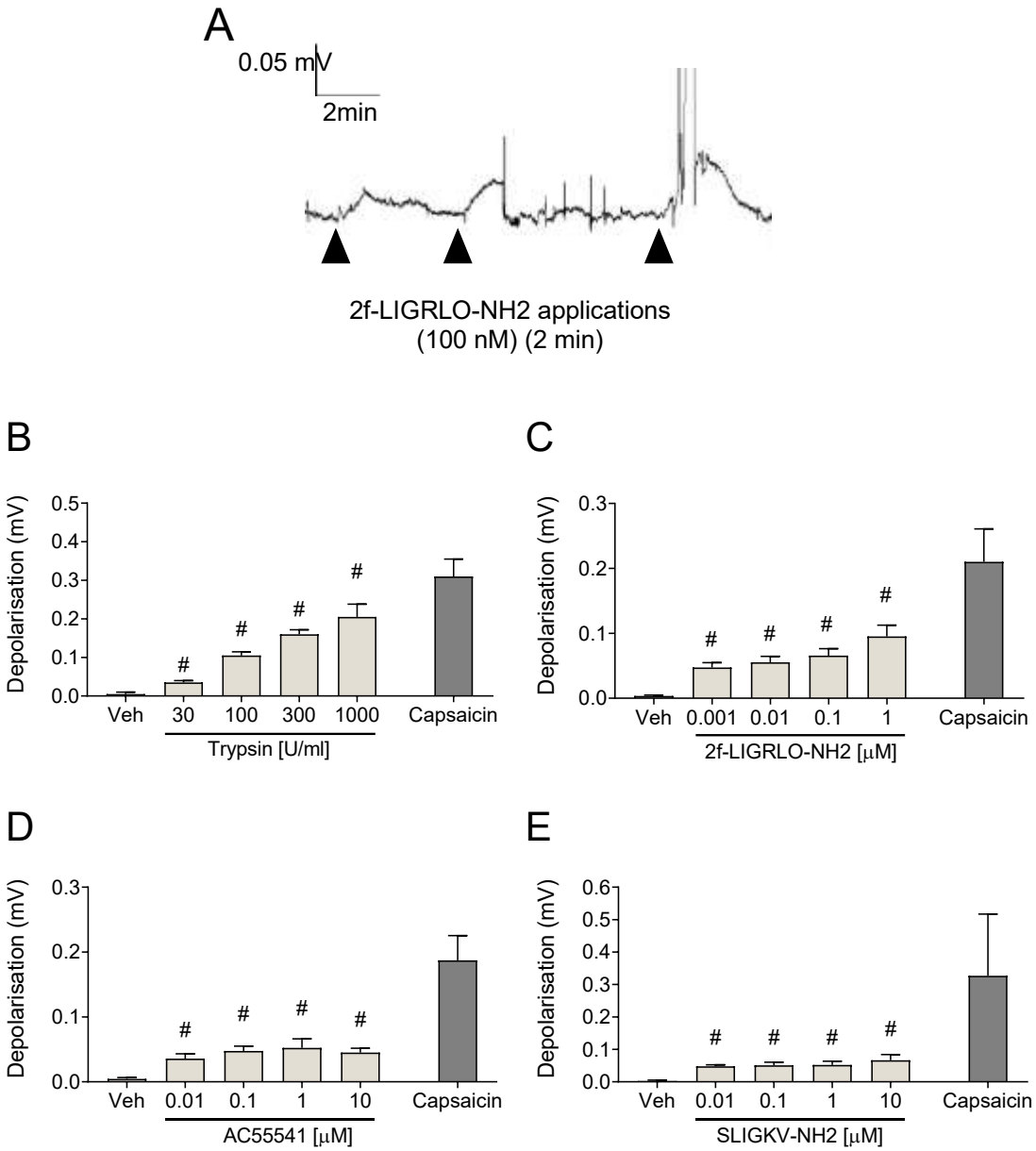


Figure 6-6 Effect of PAR2 agonists on vagus depolarisation

Rat isolated vagal nerves were stimulated with the vehicle (0.1% H_2O or 0.1% DMSO) followed by increasing concentrations of the PAR2 agonist trypsin, 2f-LIGRLO-NH2, SLIGKV-NH2 or AC55541, with each concentration applied for 2 min. Trypsin (30 - 1000 U/ml) corresponded to ~ (500 nM - 16 μM). The TRPV1 agonist capsaicin (1 μM) was applied at the end of each experiment as a reference stimulus and to assess nerve viability. **(A)** Representative trace of 2f-LIGRLO-NH2 (100 nM) induced depolarisation of rat isolated vagus nerve. **(B, C, D, E)** Quantification of the depolarisation (mV) induced by the PAR2 agonists and by the reference stimulus capsaicin. Data expressed as mean ± S.E.M., N=4-8 rats. One-way ANOVA followed by Dunnett's multiple comparisons $^{\#}p<0.05$ vs vehicle response.

6.5.3 Effect of the PAR2 agonist 2f-LIGRLO-NH2 on *Par2*$^{-/-}$ mouse vagus depolarisation

To confirm that 2f-LIGRLO-NH2 was depolarising rat vagal nerves via activation of PAR2 receptors, its effect was studied on vagal nerves isolated from *Par2*$^{-/-}$ mice. The genotypes of *Par2*$^{-/-}$ and *Par2*$^{+/+}$ mice were confirmed by PCR as represented in (**Figure 6-7**).

Incubation with 2f-LIGRLO-NH2 (100 nM) depolarised vagal nerves isolated from *Par2*$^{+/+}$ mice (0.03 ± 0.01 mV vs 0.17 ± 0.04 mV) and the effect was lost in *Par2*$^{-/-}$ mice (0.02 ± 0.01 mV vs 0.02 ± 0.01 mV) compared to the vehicle (0.1% DMSO). As a positive control, TRPV4 agonist GSK1016790A (300 nM) and TRPV1 agonist capsaicin (1 µM) responses were unchanged in nerves isolated from *Par2*$^{-/-}$ versus *Par2*$^{+/+}$ mice (**Figure 6-7**).

6.5.4 Effect of the PAR2 antagonist P2pal-18S on PAR2-induced rat vagus depolarisation

P2pal-18S was selected as a PAR2 antagonist to confirm that 2f-LIGRLO-NH2-induced rat vagus depolarisation was mediated via PAR2. At the same time, this enabled to validate P2pal-18S as a suitable antagonist for blocking PAR2 nerve responses in the rat species. P2pal-18S is a pepducin lipopeptide that comprises a peptide sequence mimicking PAR2 intracellular loops and a palmitate lipid that renders the compound cell permeable. After translocation across the cell membrane, the pepducin binds to PAR2 intracellular domains and prevents interactions with G proteins (M. K. Yau, Liu, and Fairlie 2013; M. S. Lee and Lerner 2019).To account for non-specific effects, a Scrambled pepducin comprising the same aminoacids but in a randomised order was custom-made and tested alongside.

P2pal-18S (10 µM) significantly inhibited the depolarisation induced by the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) by 83% (0.06 ± 0.01 mV vs 0.01 ± 0.01 mV) whereas the Scrambled peptide and vehicle (0.1% DMSO) had no effect (**Figure 6-8**).

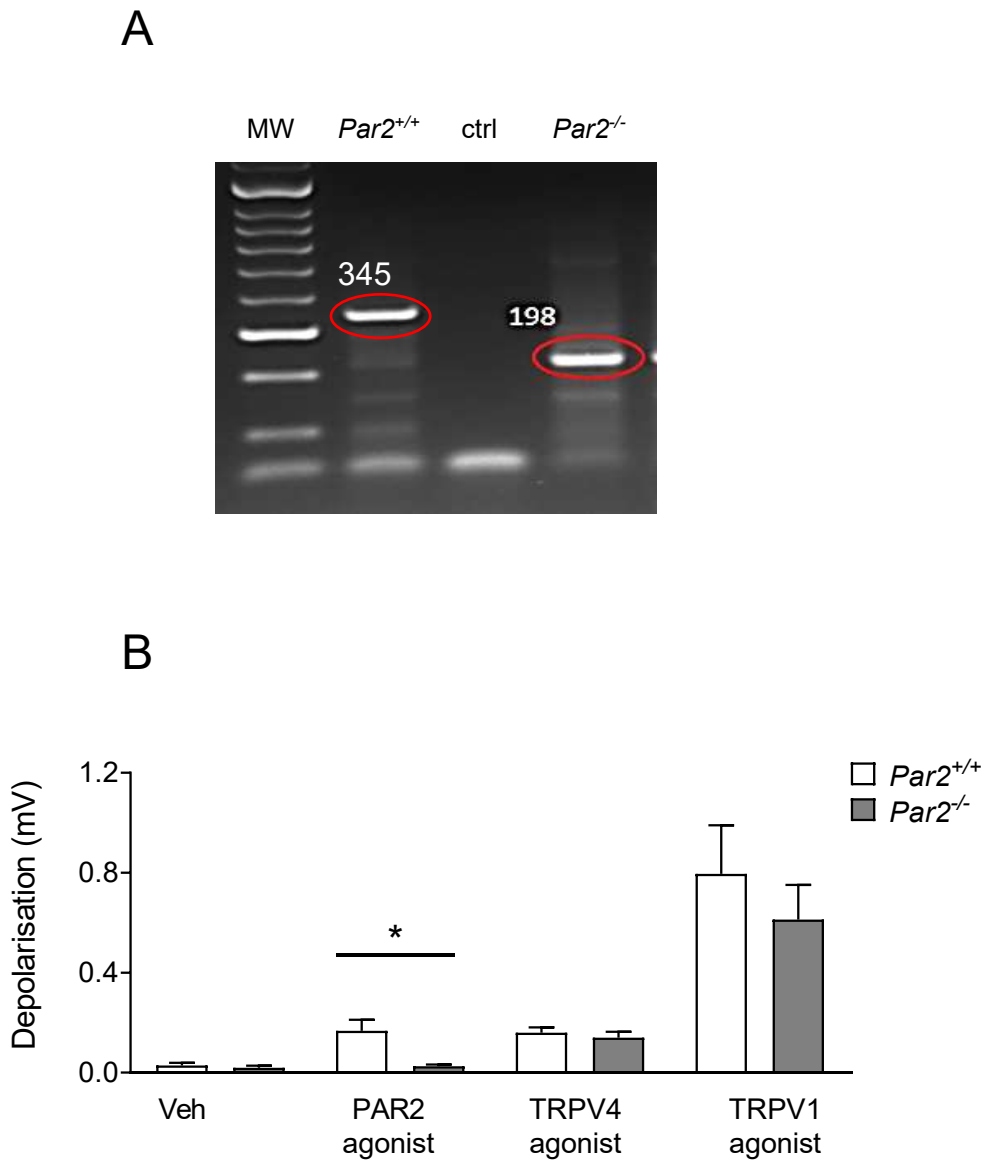


Figure 6-7 Effect of the PAR2 agonist on vagus depolarisation in *Par2*$^{-/-}$ mice

(A) Representative picture of *Par2* genotyping of the mice used in vagus experiments. PCR products from mouse tail DNA were separated by agarose gel electrophoresis. Expected amplicon size is 345-bp for *Par2*$^{+/+}$ gene and 198-bp for *Par2*$^{-/-}$ gene. MW: DNA Ladder. Bp: base pairs. Ctrl: No DNA template control. **(B)** Vagal nerves were isolated from *Par2*$^{-/-}$ and littermate *Par2*$^{+/+}$ controls. Nerves were successively stimulated with the vehicle (0.1% DMSO), PAR2 agonist 2f-LIGRLO-NH2 (100 nM), TRPV4 agonist GSK1016790A (300 nM) and TRPV1 agonist capsaicin (1 µM) applied for 2 min. The graph represents the quantification of the depolarisation (mV). Data expressed as mean ± S.E.M., N=6 mice per group. Unpaired t-test *p<0.05 *Par2*$^{-/-}$ vs *Par2*$^{+/+}$ responses.

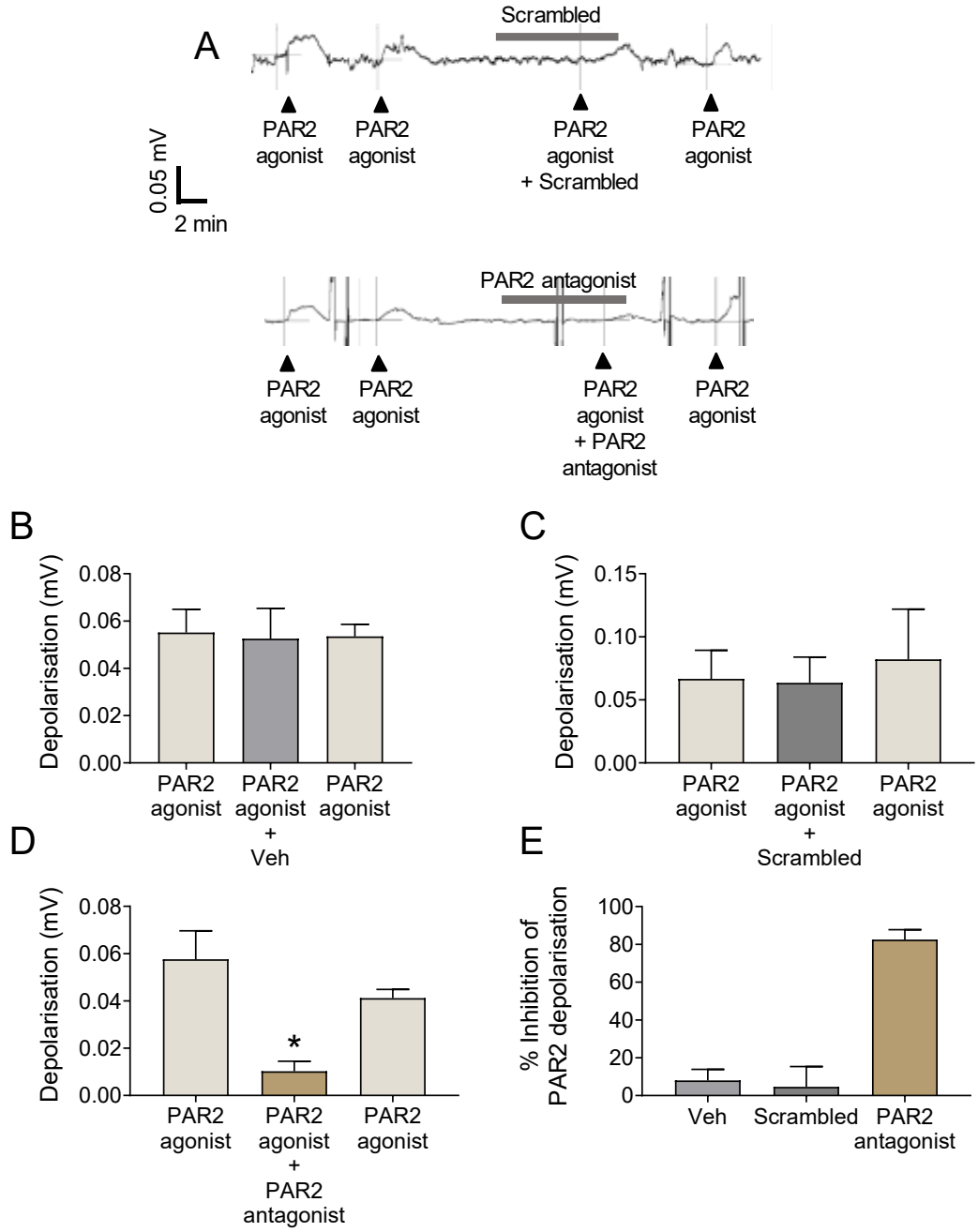


Figure 6-8 Effect of the PAR2 antagonist P2pal-18S on PAR2-induced vagus depolarisation

Rat isolated vagal nerves were stimulated with the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) and the depolarisation of the nerve assessed in absence or presence of the PAR2 antagonist P2pal-18S (10 μM), control Scrambled peptide (10 μM) or vehicle (0.1% DMSO). **(A)** Representative traces. **(B, C)** Quantification of the depolarisation (mV) induced by the PAR2 agonist (left bar), in presence of the vehicle, control peptide or antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). **(E)** Percentage inhibition of PAR2 agonist-induced depolarisation. Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *p<0.05 comparing the agonist response before and after antagonist treatment.

6.5.5 Effect of the PAR2 agonist on Aδ-fibre firing and intratracheal pressure in anaesthetised naïve rats

The ability of PAR2 to specifically activate airway sensory nerve terminals was tested *in vivo* using the single fibre preparation in anaesthetised naïve rats. The interrogated fibres presented spontaneous activity in rhythm with the breathing pattern and responded to an aerosol of citric acid (0.3 M) and to lung hyperinflation/deflation. The fibres were therefore identified as airway sensory Aδ-fibres (RARs).

In a naïve rat, nebulising increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 directly into the rat trachea provoked an increase in action potential firing of the studied Aδ-fibre at the concentration of 1 µM compared to the vehicle (0.1% DMSO) (N=1) (**Figure 6-9**). The PAR2 agonist did not provoke a rise in intratracheal pressure at any of the concentrations tested. The steady increase in intratracheal pressure over time was attributed to a rise in baseline pressure potentially due to an accumulation of fluids in the animal.

To ensure that Aδ-fibre firing was due to PAR2 activation, the single concentration of 1 µM 2f-LIGRLO-NH2 was tested against the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.) in another naïve rat. In this experiment, the PAR2 agonist 2f-LIGRLO-NH2 (1 µM) seemed to provoke Aδ-fibre firing in a reproducible manner, without increasing intratracheal pressure. Dosing the rat with P2pal-18S (10 mg/kg, s.c.) appeared to inhibit the response to 2f-LIGRLO-NH2 (1 µM) while it did not affect the response to the reference stimulus citric acid (0.3 M) (N=1) (**Figure 6-10**).

This preliminary experiment validated the use of P2pal-18S as a suitable antagonist at the concentration of 10 mg/kg, s.c. for blocking PAR2-afferent nerve firing *in vivo*.

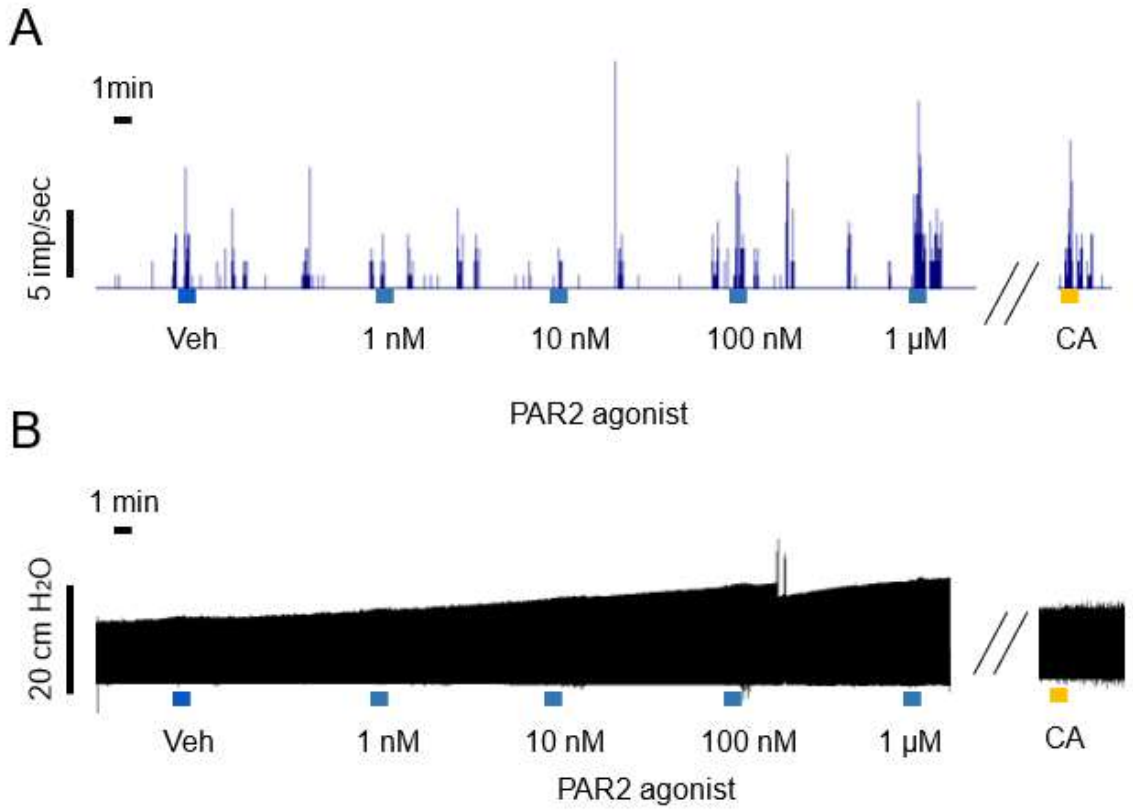


Figure 6-9 Effect of the PAR2 agonist on Aδ-fibre firing and local bronchospasm

A rat was anaesthetised, paralysed and both vagi disconnected from their central end. An airway sensory Aδ-fibre was identified and its electrical activity recorded over time. Intratracheal pressure was monitored via a pressure transducer. The vehicle (0.1% DMSO) followed by increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 were nebulised for 1 min into the rat airways via a tracheal cannula. At the end of the experiment, the reference stimulus citric acid (CA) (0.3 M) was nebulised for 1 min to assess nerve viability. **(A) F**requency of action potential firing events above baseline activity (impulses/sec) of the Aδ-fibre over time. **(B)** Intratracheal pressure (cm H_2O) over time. N=1 rat.

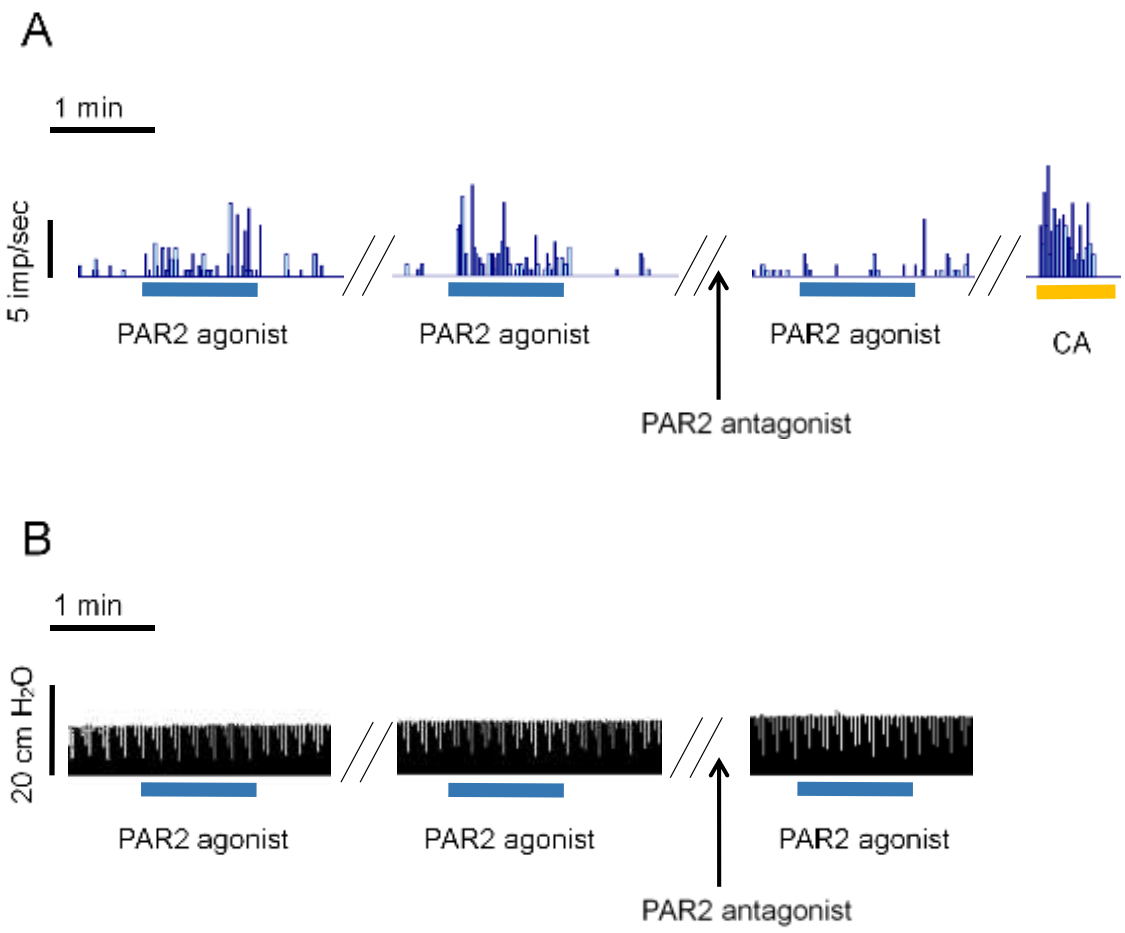


Figure 6-10 Effect of the PAR2 antagonist on PAR2-mediated Aδ-fibre firing and local bronchospasm

A rat was anaesthetised, paralysed and both vagi disconnected from their central end. An airway sensory Aδ-fibre was identified and its electrical activity recorded over time. Intratracheal pressure was monitored via a pressure transducer. The PAR2 agonist 2f-LIGRLO-NH2 (1 μM) was nebulised for 1 min. This was repeated to ensure reproducibility of the response. The rat was then dosed with the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.). After 30 min, the PAR2 agonist was nebulised for 1 min. At the end of the experiment, the reference stimulus citric acid (CA) (0.3 M) was nebulised for 1 min to assess nerve viability. **(A)** Frequency of action potential firing events above baseline activity (impulses/sec) of the Aδ-fibre over time. **(B)** Intratracheal pressure (cm H_2O) over time. N=1 rat.

6.5.6 Effect of TRPV4 and P2X3 antagonists on PAR2-induced rat vagus depolarisation

To study whether PAR2 induced the depolarisation of rat vagal nerves via the TRPV4-P2X3 axis, the TRPV4 and P2X3-P2X2/3 antagonists were tested in the vagus preparation generated from naïve rats. The TRPV4 antagonist GSK2193874 (10 µM) significantly inhibited the depolarisation induced by the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) by 95% (0.04 ± 0.01 mV vs 0.01 ± 0.01 mV). The P2X3-P2X2/3 antagonist AF-353 (10 µM) also significantly reduced the depolarisation by 58% (0.06 ± 0.01 mV vs 0.03 ± 0.01 mV). The vehicle (0.1% DMSO) had no effect (**Figure 6-11**).

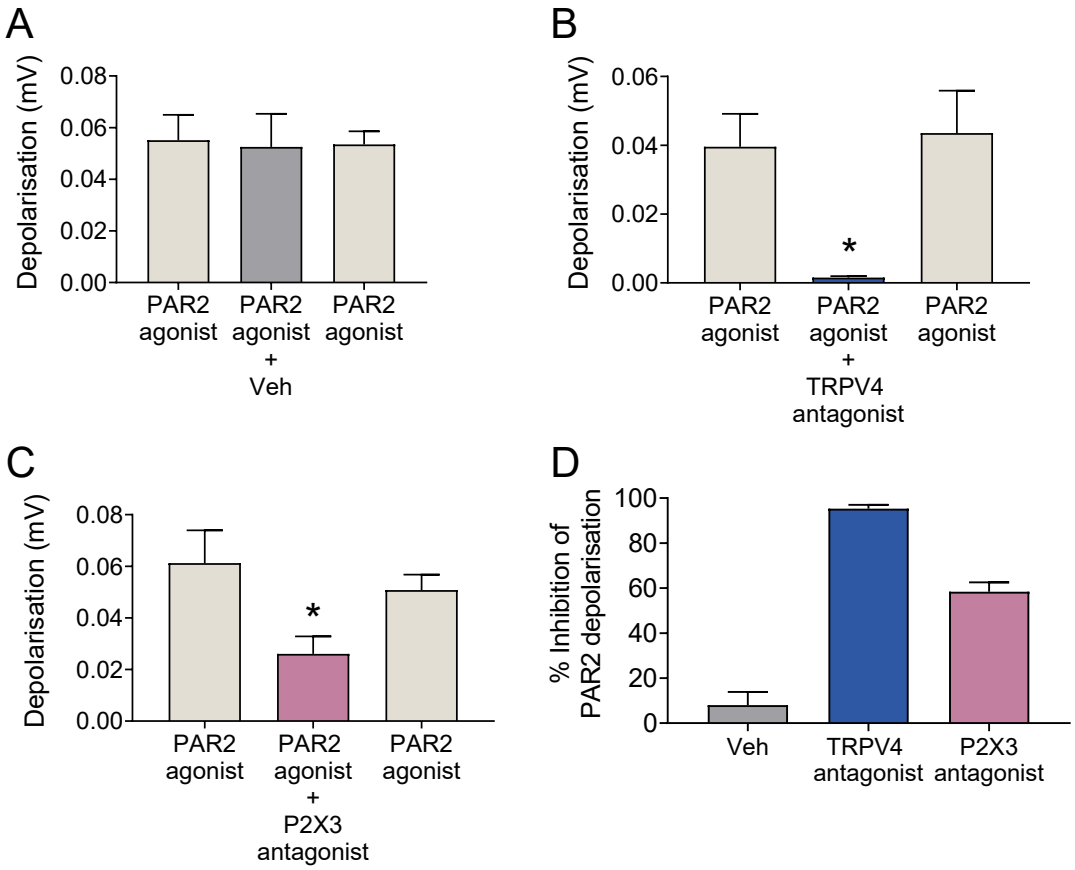


Figure 6-11 Effect of TRPV4 and P2X3 antagonists on PAR2-induced vagus depolarisation

Rat isolated vagal nerves were stimulated with the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) and the depolarisation of the nerve assessed in absence or presence of the TRPV4 antagonist GSK2193874 (10 µM), P2X3-P2X2/3 antagonist AF-353 (10 µM) or vehicle (0.1% DMSO). **(A, B, C)** Quantification of the depolarisation (mV) induced by the PAR2 agonist (left bar), in presence of the antagonist (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). **(D)** Percentage inhibition of PAR2 agonist-induced depolarisation. Data expressed as mean ± S.E.M., N=4 rats. Paired t-test *p<0.05 comparing the agonist response before and after antagonist treatment.

6.5.7 Effect of the PAR2 agonist on isolated trachea contraction

The third aim of this chapter was to investigate if PAR2 activation can provoke bronchospasm in naïve rats and if it is mediated via a reflex and/or local bronchospasm.

Preliminary results from the single fibre preparation suggested that PAR2 activation did not trigger local bronchospasm independently from a nerve reflex (**6.5.5**). To confirm this finding, the effect of the PAR2 agonist was tested *in vitro* on the contraction of rat isolated airways.

To test the effect of PAR2 activation on extrapulmonary airways contraction, a non-cumulative concentration-response of the PAR2 agonist 2f-LIGRLO-NH2 was conducted on tracheas isolated from naïve rats.

None of the concentrations tested of the PAR2 agonist 2f-LIGRLO-NH2 (10 nM to 1µM) contracted rat tracheas compared to the vehicle (0.1% DMSO) (**Figure 6-12**).

6.5.8 Effect of the PAR2 agonist on small airways contraction in PCLS

To test the effect of PAR2 activation on intrapulmonary airways contraction, a non-cumulative concentration-response of the PAR2 agonist 2f-LIGRLO-NH2 was conducted on small airways using PCLS generated from naïve rats.

None of the concentrations tested of the PAR2 agonist 2f-LIGRLO-NH2 (10 nM to 1µM) contracted rat small airways compared to the vehicle (0.1% DMSO) (**Figure 6-13**).

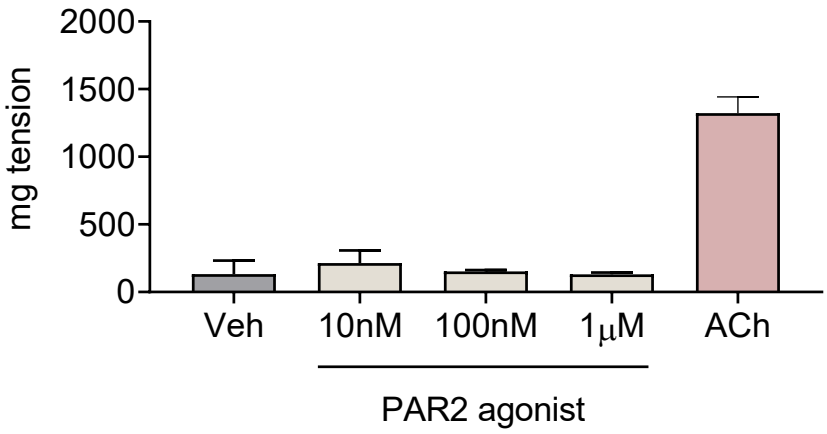


Figure 6-12 Effect of the PAR2 agonist on isolated trachea contraction

Rat isolated trachea segments were incubated with ACh (1 mM) to determine viability and maximum contractility. After washing, segments were incubated with the vehicle (0.1% DMSO) or a single concentration of the PAR2 agonist 2f-LIGRLO-NH2 and contraction followed for 40 min. Experiment conducted in presence of indomethacin (10 μM). The graph represents the maximum contraction (mg tension) elicited by the drug. N=3 rats. Data expressed as mean ± S.E.M. Kruskal-Wallis test non-significant between vehicle and agonist responses on data normalised to ACh response.

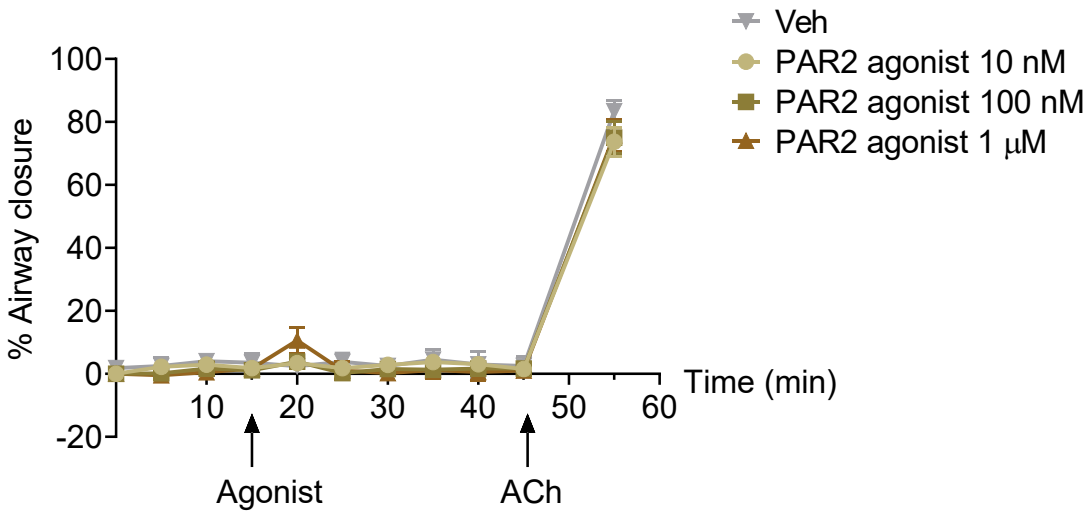


Figure 6-13 Effect of the PAR2 agonist on small airways contraction

PCLS were isolated from naïve rats and treated with the vehicle (0.1% DMSO) or a single concentration of the PAR2 agonist 2f-LIGRLO-NH2 for 30 min. ACh (1 mM) was applied at the end to assess viability and maximum contractility. Small airways contraction was assessed by light microscopy. The graph represents the trace of small airways contraction over time. Data expressed as mean ± S.E.M. N=4 rats, n=3-5 airways/rat. Kruskal-Wallis test non-significant between vehicle and agonist responses on data normalised to ACh response.

6.5.9 Effect of the PAR2 agonist on Penh in conscious naïve rats

The ability of PAR2 activation to provoke reflex bronchospasm was tested in conscious naïve rats using FWBP. Given the absence of effect on isolated airways, it was hypothesised that if PAR2 activation elicited a Penh response, it would reflect a reflex bronchospasm.

This hypothesis was tested by aerosolising increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 (0.01 – 10 µM) into the airways of conscious naïve rats. To reduce the number of animals, this was tested in a cumulative manner in the same rats. To account for a possible effect of solvent accumulation and rise in humidity, a second group of animals received time-matched vehicles (0.00001% - 0.1% DMSO).

Aerosolising increasing concentrations of the PAR2 agonist 2f-LIGRLO-NH2 did not increase Penh AUC compared to time-matched vehicles (**Figure 6-14**).

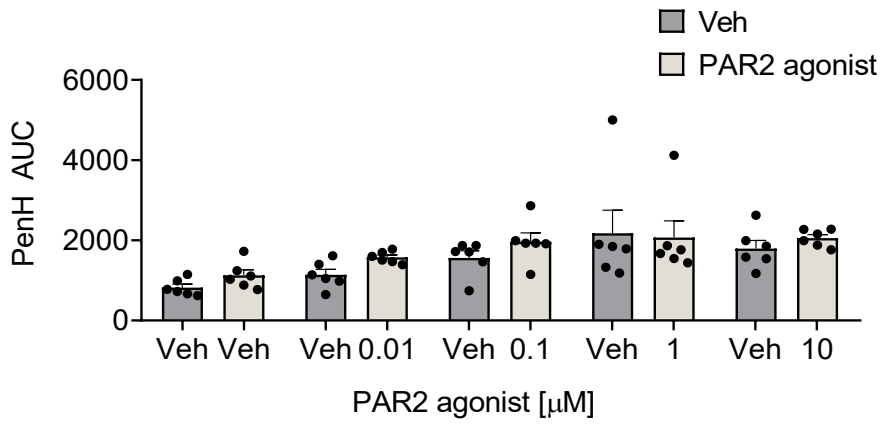


Figure 6-14 Effect of the PAR2 agonist on Penh in conscious naive rats

Conscious naïve rats were challenged with increasing concentrations of either the vehicle (0.00001% - 0.1% DMSO) or PAR2 agonist 2f-LIGRLO-NH2 (0.01 – 10 µM) with each concentration aerosolised for 10 min and Penh recorded for 20 min using FWBP. The graph represents the quantification of Penh AUC, N=6/group. Data expressed as mean ± S.E.M. Mann-Whitney test non-significant between agonist and time-matched vehicle challenges.

6.5.10 Effect of the PAR2 antagonist P2pal-18S on the LAR in the rat OVA model

The final aim of this chapter was to test whether PAR2 receptors were involved in driving the LAR in the rat OVA model, using the pepducin PAR2 antagonist P2pal-18S.

The PAR2 antagonist P2pal-18S was tested against the LAR in the rat OVA model. The Scrambled peptide was tested alongside to account for non-specific effects of the antagonist, as well as the solvent (5% DMSO) itself.

OVA challenge increased Penh AUC compared to SAL challenge in OVA-sensitised rats (24177 ± 1279 vs 143423 ± 31008 Penh AUC). The PAR2 antagonist P2pal-18S (10 mg/kg, s.c.) did not prevent this Penh increase compared to the vehicle (143423 ± 31008 vs 185184 ± 43059 Penh AUC). However, the control Scrambled peptide (10 mg/kg, s.c.) seemed to prevent the increase in Penh caused by OVA challenge compared to the vehicle (143423 ± 31008 vs 63176 ± 30044 Penh AUC) although this was not statistically significant (**Figure 6-15**).

6.5.11 Effect of the PAR2 antagonist P2pal-18S on airway inflammation in the rat OVA model

In addition to bronchospasm, OVA challenge provoked airway inflammation compared to SAL challenge in OVA-sensitised rats, with an increase in total leukocytes (162.5 ± 19 vs 646.9 ± 207 $.10^3$ cells/ml), eosinophils (5.3 ± 1.9 vs 52.4 ± 9.9 $.10^3$ cells/ml) and neutrophils (15.0 ± 3.4 vs 461.5 ± 183 $.10^3$ cells/ml) numbers in BAL fluid at 24 h post-challenge. The PAR2 antagonist P2pal-18S did not significantly reduce airway inflammation, nor did the control Scrambled peptide (**Figure 6-16**).

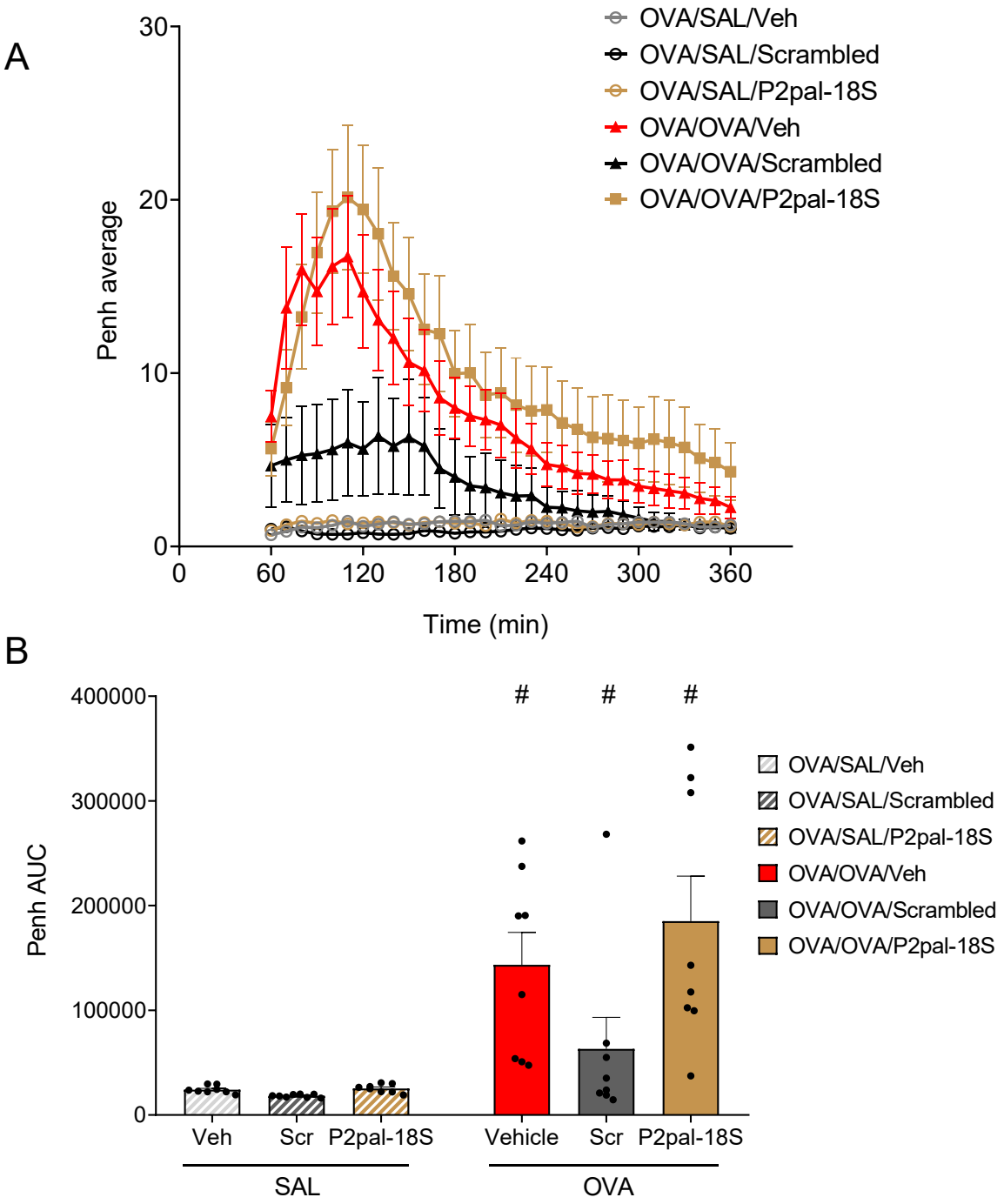


Figure 6-15 Effect of the PAR2 antagonist P2pal-18S on the LAR in the rat OVA model

Rats were sensitised with OVA and challenged with OVA or SAL aerosol. Rats were dosed with the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.), control Scrambled (Scr) peptide (10 mg/kg, s.c.) or vehicle (5% DMSO, s.c.) 1 h before and 30 min after challenge. Penh was assessed 1 to 6 h post-challenge via FWBP. **(A)** Penh average over time. **(B)** Quantification of Penh AUC over the 5 h-recording period. Data expressed as mean ± S.E.M., N=8 rats per group. Mann-Whitney test $^{\#}p<0.05$ OVA challenge vs respective SAL challenge, and non-significant vs OVA/OVA/Veh.

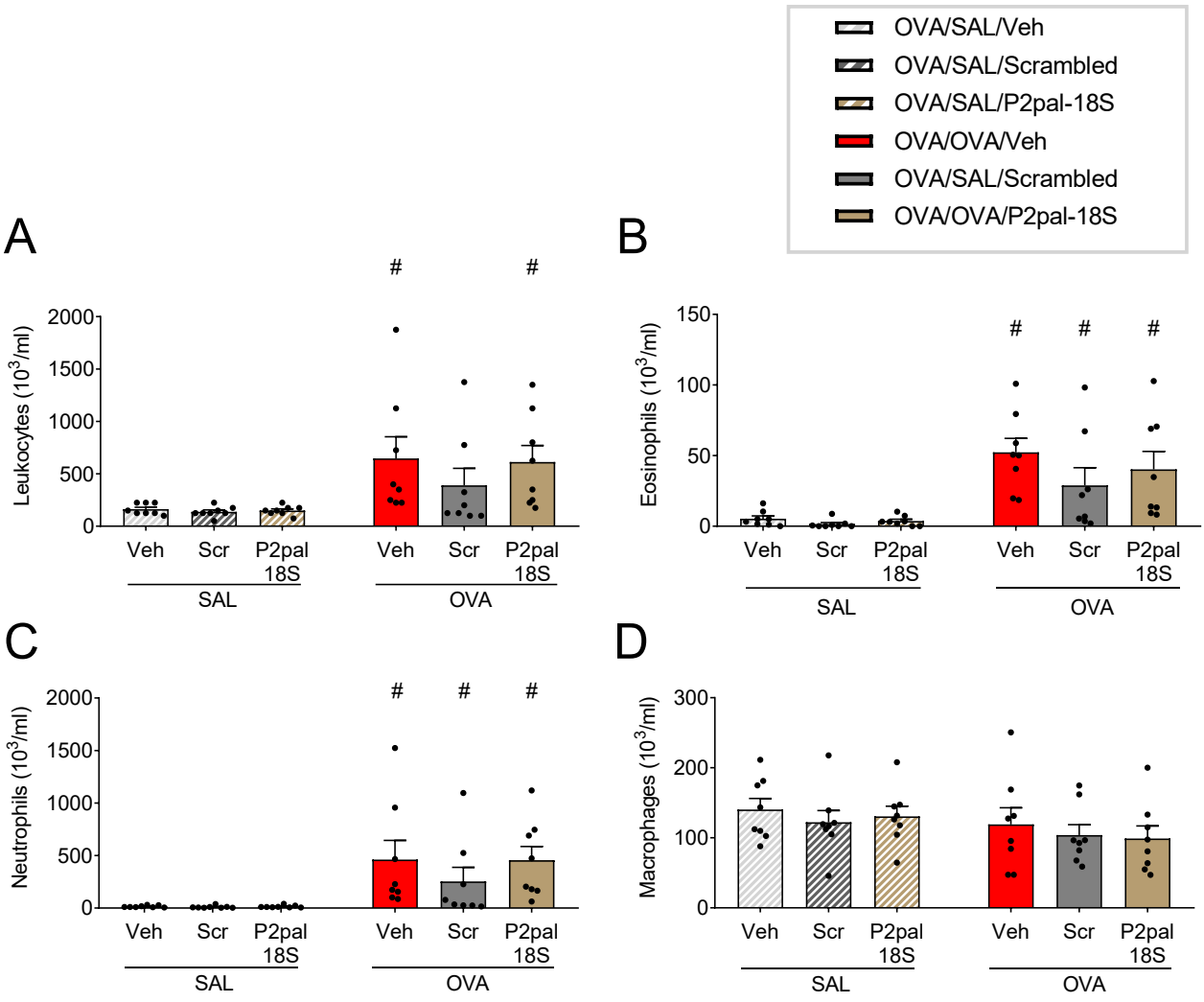


Figure 6-16 Effect of the PAR2 antagonist P2pal-18S on airway inflammation in the rat OVA model

Rats were sensitised with OVA and challenged with OVA or SAL aerosol. Rats were dosed with the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.), control Scrambled (Scr) peptide (10 mg/kg, s.c.) or vehicle (5% DMSO, s.c.) 1 h before and 30 min after challenge. Airway inflammation was assessed in BAL at 24 h post-challenge by differential cell count: **(A)** Total leukocytes, **(B)** Eosinophils, **(C)** Neutrophils and **(D)** Monocytes/Macrophages. Data expressed as mean ± S.E.M., N=8 rats per group. Mann-Whitney test $^{\#}p<0.05$ OVA challenge vs respective SAL challenge, and non-significant vs OVA/OVA/Veh. Legend in box above the graphs.

6.5.12 Validation of the compounds used in the rat OVA model

Due to the unexpected nature of the results, drug solutions used in the study were carefully examined. Solutions were sent for chemical analysis and peptide sequencing revealed that P2pal-18S and Scrambled-labelled solutions contained peptides with the appropriate sequence. In addition, solutions were tested in the laboratory for their biological activity against PAR2-induced rat vagus depolarisation at the appropriate dilution (10 µM). As shown in (**Figure 6-17**), the P2pal-18S-labelled solution inhibited the depolarisation provoked by the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) while the Scrambled peptide-labelled solution did not. These assays confirmed that drugs solutions used in the study were not switched.

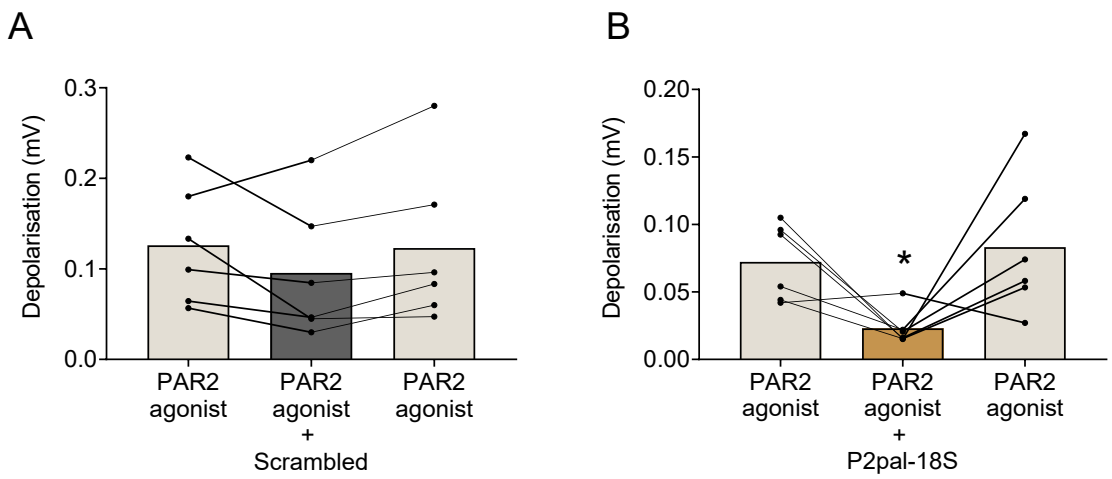


Figure 6-17 Activity of the drug solutions against PAR2-induced vagus depolarisation

Vagal nerves were isolated from naïve rats and incubated with the PAR2 agonist 2f-LIGRLO-NH2 (100 nM) to induce nerve depolarisation. Nerves were incubated with **(A)** the Scrambled peptide-labelled solution and **(B)** the P2pal-18S-labelled solution, at adjusted dilutions to obtain a final concentration of 10 µM. Graphs represent the quantification of the depolarisation (mV) induced by the PAR2 agonist (left bar), in presence of the peptide solution (middle bar). Recovery of the agonist response was assessed at the end of each experiment (right bar). Data expressed as mean with each line representing an experiment in one animal. N=6 rats. Paired t-test *$p<0.05$ comparing the agonist response before and after peptide incubation.

6.5.13 Repetition of the PAR2 antagonist P2pal-18S study in the rat OVA model

As drug solutions made for the study contained the correct peptides, the study was repeated to confirm these findings. As a positive control, the TRPV4 antagonist GSK2193874 was included alongside in the rat OVA model. A new batch of P2pal-18S and Scrambled peptide were synthesised, sequenced and tested against PAR2 agonist-induced depolarisation of rat vagal nerves. P2pal-18S (10 µM) inhibited the depolarisation induced by 2f-LIGRLO-NH2 (100 nM) (data not shown).

In this study, OVA challenge resulted in an increase in Penh in OVA-sensitised rats. The positive control TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) diminished this increase in Penh. The PAR2 antagonist P2pal-18S (10 mg/kg, s.c.) had no effect on OVA-induced Penh increase, however the Scrambled peptide (10 mg/kg, s.c.) significantly reduced it compared to the vehicle, corroborating findings from the first study (**Figure 6-18**).

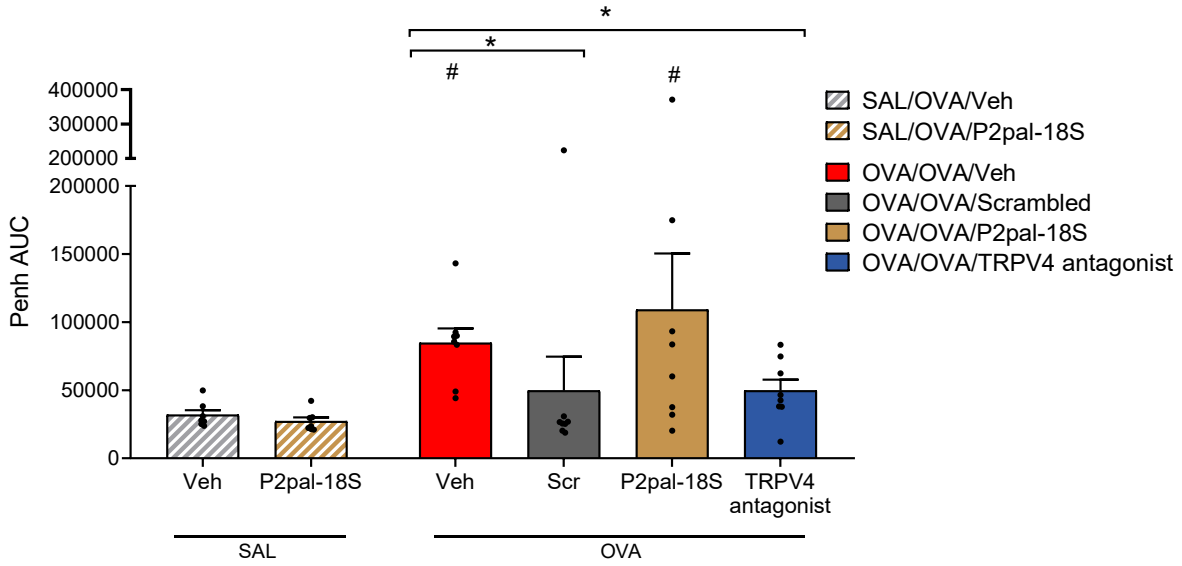


Figure 6-18 Repeat: effect of the PAR2 antagonist P2pal-18S on the LAR in the rat OVA model
Rats were sensitised with OVA or SAL and challenged with OVA aerosol. Rats were dosed with the PAR2 antagonist P2pal-18S (10 mg/kg, s.c.), control Scrambled (Scr) peptide (10 mg/kg, s.c.), TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) or vehicle (5% DMSO, s.c. and 6% HP-β-cyclodextrin, i.p.) 1 h before and 30 min after challenge. Penh was assessed 1 to 6 h post-challenge via FWBP. The graph represents the quantification of Penh AUC over the 5 h-recording period. Data expressed as mean ± S.E.M., N=7-8 rats per group. Mann-Whitney test $^{\#}p<0.05$ OVA challenge vs respective SAL challenge and *p<0.05 vs OVA/OVA/Veh.

6.5.14 Effect of the anti-PAR2 monoclonal antibody MEDI2344 on the LAR in the rat OVA model

Seeing these results, another approach was used to block PAR2 signalling in the rat OVA model, by testing the anti-PAR2 monoclonal antibody (mAb) MEDI2344. By binding to PAR2 receptors, this monoclonal IgG antibody prevents the proteolytic cleavage that unmasks the tethered ligand and therefore inhibits subsequent PAR2 signalling. To account for non-specific effects of the antibody, the isotype control IgG R347 was used alongside, as well as the solvent itself (25 mM histidine, 205 mM sucrose or “His-sucrose”).

In this study, OVA challenge increased Penh AUC compared to SAL challenge in OVA-sensitised rats (25448 ± 758 vs 116081 ± 33986 Penh AUC). The anti-PAR2 monoclonal antibody MEDI2344 (10 mg/kg, s.c.) did not prevent this Penh increase compared to the isotype control (194686 ± 48896 vs 191823 ± 43730 Penh AUC) (**Figure 6-19**).

6.5.15 Effect of the anti-PAR2 monoclonal antibody MEDI2344 on airway inflammation in the rat OVA model

In addition to bronchospasm, OVA challenge provoked airway inflammation compared to SAL challenge in OVA-sensitised rats, with an increase in total leukocytes (170.8 ± 23.6 vs 460.7 ± 75.8 $.10^3$ cells/ml), eosinophils (0.2 ± 0.2 vs 51.8 ± 12.3 $.10^3$ cells/ml) and neutrophils (0.0 ± 0.0 vs 272.4 ± 71.1 $.10^3$ cells/ml) numbers in BAL fluid at 24 h post-challenge. The anti-PAR2 antagonist P2pal-18S did not significantly reduce airway inflammation compared to the isotype control IgG (**Figure 6-20**).

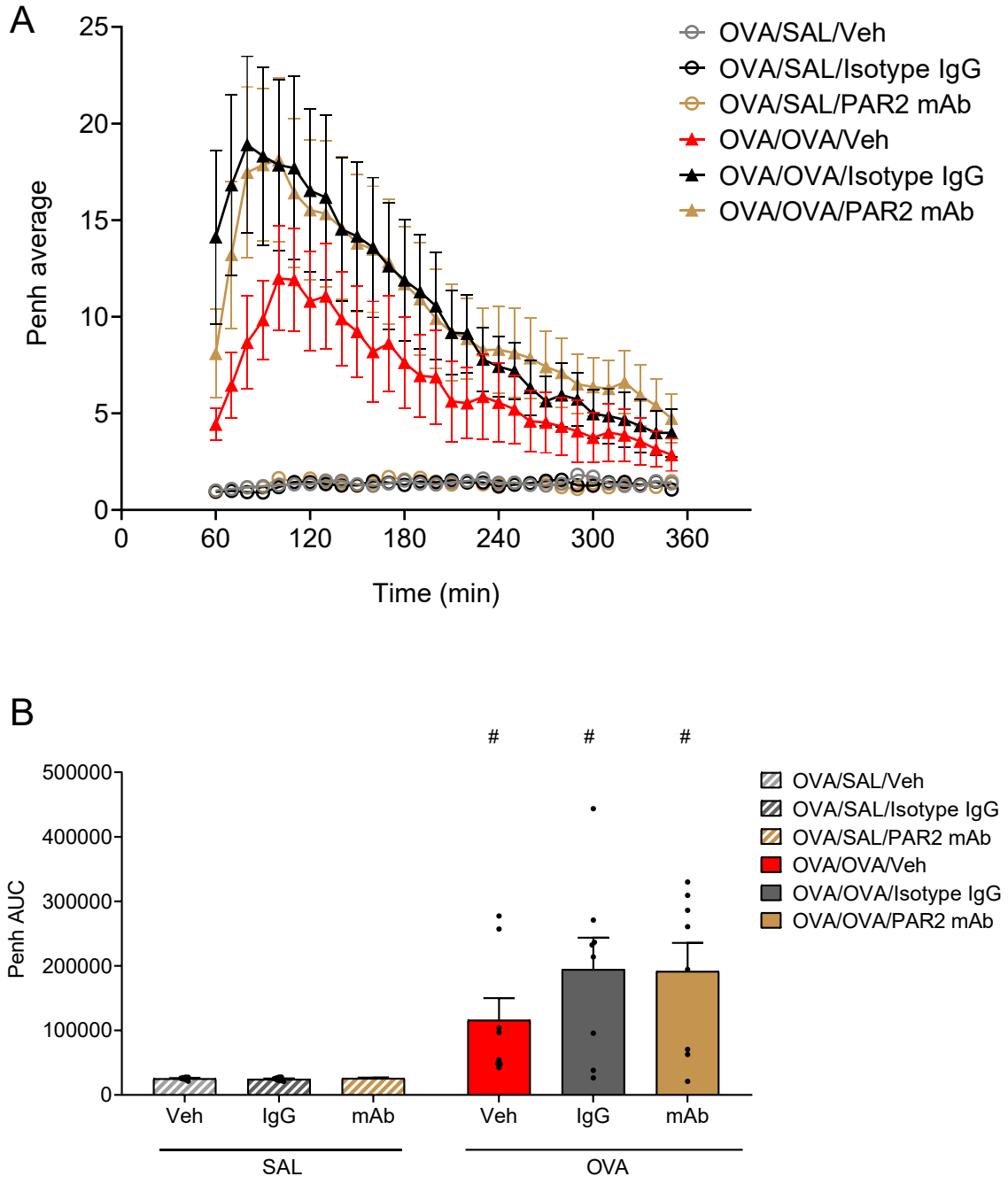


Figure 6-19 Effect of the anti-PAR2 mAb MEDI2344 on the LAR in the rat OVA model

Rats were sensitised with OVA and challenged with OVA or SAL aerosol. Rats were dosed with the anti-PAR2 mAb MEDI2344 (mAb) (10 mg/kg, s.c.), isotype IgG control (R347) (10 mg/kg, s.c.) or vehicle (His-sucrose, s.c.) one day before challenge. Penh was assessed 1 to 6 h post-challenge via FWBP. **(A)** Penh average over time. **(B)** Quantification of Penh AUC over the 5 h-recording period. Data expressed as mean ± S.E.M., N=8 rats per group. Mann-Whitney test $^{\#}p<0.05$ OVA challenge vs respective SAL challenge, and non-significant vs OVA/OVA/Veh.

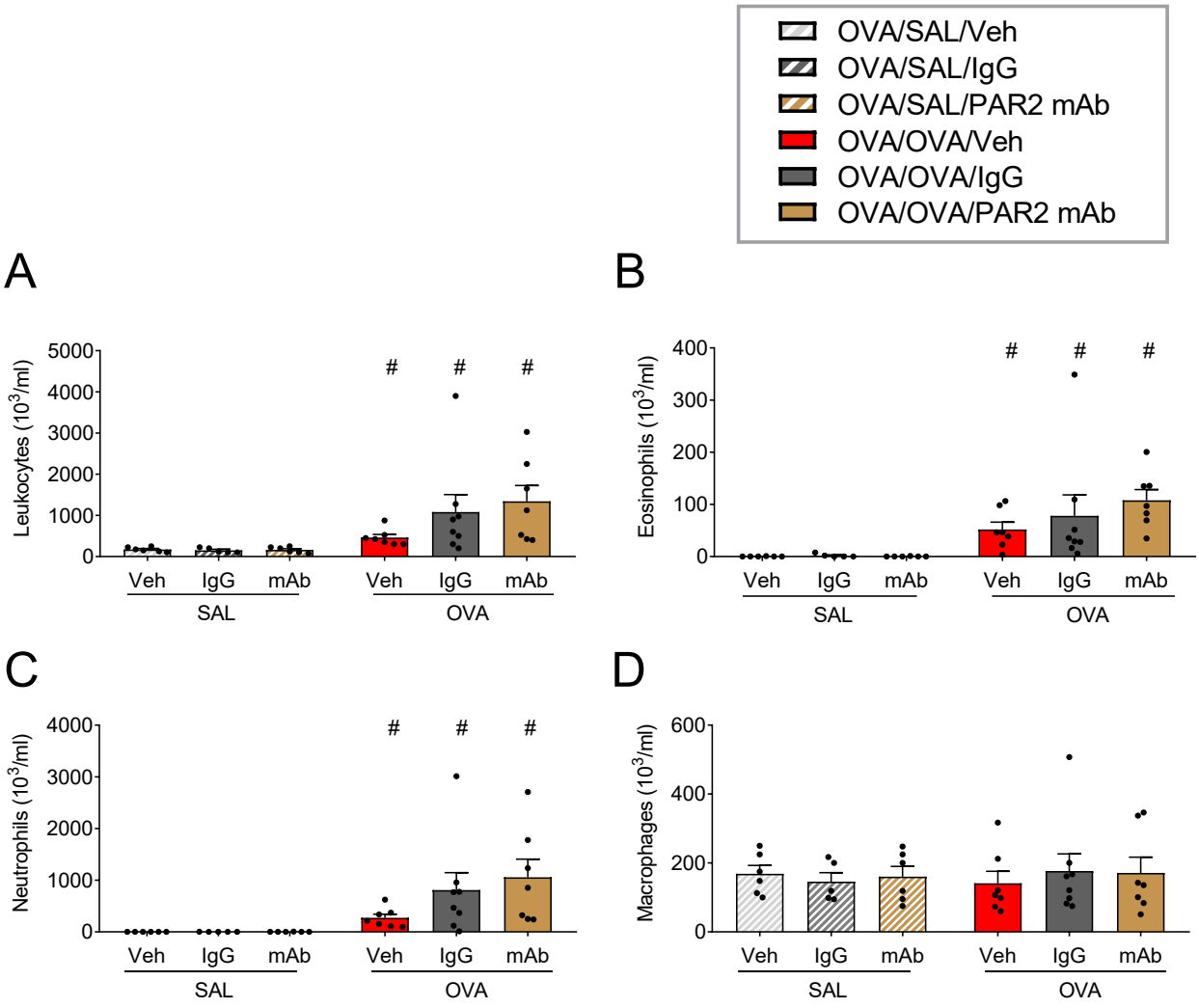


Figure 6-20 Effect of the anti-PAR2 mAb MEDI2344 on airway inflammation in the rat OVA model
Rats were sensitised with OVA and challenged with OVA or SAL aerosol. Rats were dosed with the anti-PAR2 mAb MEDI2344 (mAb) (10 mg/kg, s.c.), isotype IgG control (R347) (10 mg/kg, s.c.) or vehicle (His-sucrose, s.c.) one day before challenge. Airway inflammation was assessed in BAL at 24 h post-challenge by differential cell count: **(A)** Total leukocytes, **(B)** Eosinophils, **(C)** Neutrophils and **(D)** Monocytes/Macrophages. Data expressed as mean ± S.E.M., N=5-8 rats per group. Mann-Whitney test $^{\#}p<0.05$ OVA challenge vs respective SAL challenge, and non-significant vs OVA/OVA/Veh. Legend in box above the graphs.

6.6 Discussion

In **Chapter 5**, the TRPV4 agonist did not seem to provoke reflex bronchospasm in naïve rats. Given the sustained afferent nerve firing provoked by TRPV4 activation in the rat (**Chapter 3**) and guinea pig (Bonvini et al. 2016) and the important blockade of the LAR by the TRPV4 and P2X3-P2X2/3 antagonists in the rat OVA model (**Chapter 4**), it was hypothesised that a TRPV4-P2X3 afferent nerve signal could elicit a reflex in the context of allergic airway inflammation but not in the naïve state. To study this hypothesis, this chapter investigated how the TRPV4-P2X3 nerve axis could be activated in the LAR. PAR2 receptors were considered as promising candidates for three main reasons:

- 1) PAR2 receptors have been shown to activate and/or sensitise TRPV4 channels, eliciting a signal in spinal sensory neurons and an efferent response in the context of hyperalgesia in the somatosensory and visceral systems (Cenac et al. 2015; Grant et al. 2007; Poole et al. 2013; Sipe et al. 2008; Zhao et al. 2014; 2015), highlighting PAR2 receptors as possible activators of a TRPV4-nerve reflex in presence of inflammation.

- 2) PAR2 receptors have been involved in allergic airway disease: PAR2 receptors and several PAR2 agonists were found upregulated in the airways of asthmatic patients at baseline (Aubier et al. 2016; Allard et al. 2014; Knight et al. 2001; Jarjour et al. 1991; M. J. Alvarez et al. 2000; S. E. Wenzel, Fowler, and Schwartz 1988) and several mouse models have showed that PAR2 blockade or deletion reduces allergic airway inflammation and AHR (Schmidlin et al. 2002; Davidson et al. 2013; de Boer et al. 2014; Asaduzzaman et al. 2015, 2018).

- 3) Allergen challenge is associated with the release of the potent PAR2 agonist tryptase in the BAL fluid of asthmatic patients (S. E. Wenzel, Fowler, and Schwartz 1988) and tryptase inhibitors have reduced the LAR in human and animal models (Krishna et al. 2001; Costanzo et al. 2003; Clark et al. 1995), highlighting a potential role for PAR2 receptors in the LAR.

Before testing this hypothesis, it was important to evaluate whether the rat OVA model replicated the upregulation of PAR2 agonists seen in asthmatic patients (Knight et al. 2001; Aubier et al. 2016; S. E. Wenzel, Fowler, and Schwartz 1988). To evaluate levels of PAR2 agonists, an enzymatic assay measured the levels of tryptase-like activity in BAL samples after OVA challenge. Unlike classical GPCRs, PAR2 receptors are activated by proteases which cleave their N-terminus, thereby revealing a ligand on the receptor itself (Adams et al. 2011). Many proteases have been shown to cleave PAR2 receptors, including tryptase, trypsin

and coagulation factors which cleave the receptor at the same canonical site and trigger G protein signalling (G_q, G_i, $G_{12/13}$). Other proteases such as cathepsin G, S or neutrophil elastase have been shown to cleave PAR2 at other sites, triggering different G protein signalling or disarming the receptor to further activation by other proteases, a phenomenon known as “biased signalling” (Heuberger and Schuepbach 2019; Hollenberg et al. 2014; Adams et al. 2011). In this thesis, tryptase was considered as a relevant PAR2 agonist for activating a nerve reflex given that tryptase inhibitors alleviated the LAR in human and animal studies (Clark et al. 1995; Costanzo et al. 2003; Krishna et al. 2001). Furthermore, tryptase is presumably released by mast cells upon allergen challenge (S. E. Wenzel, Fowler, and Schwartz 1988; Sedgwick et al. 1991; Bradding and Arthur 2016) and many reports have highlighted a cross-talk between mast cells and sensory nerves in allergic airway disease (Undem and Taylor-Clark 2014).

Due to this interest in tryptase and proteases that could trigger similar PAR2 signalling, tryptase-like activity was evaluated in BAL samples in the rat model by measuring the cleavage of the substrate tosyl-gly-pro-lys-*p*NA (Schwartz et al. 1987). Tryptase-like activity was upregulated after OVA challenge from 2 to 24 h in the BAL of OVA-sensitised rats, suggesting that tryptase-like proteases are elevated after allergen challenge and could potentially activate PAR2 receptors, although it is possible that this enzymatic assay also captured non tryptase-like mechanisms as the substrate is not specific. In human, tryptase has been found elevated in BAL fluid immediately after allergen challenge in asthmatic patients (S. E. Wenzel, Fowler, and Schwartz 1988), however, data lacks on further timepoints. It is usually cited that tryptase levels decrease during the LAR but this assumption is mostly based on a study measuring tryptase in BAL at 48 h after allergen challenge in allergic rhinitis patients (Sedgwick et al. 1991).

These indications of an upregulation of PAR2 signalling after OVA challenge validated the use of the rat OVA model to study PAR2 in the LAR. Therefore, the second aim of this chapter addressed whether PAR2 receptors can stimulate airway sensory nerves in naïve rats. As it was the first time that PAR2 was investigated in the laboratory, concentration-responses of a range of PAR2 agonists were conducted on the depolarisation of rat isolated vagal nerves, a preparation allowing to perform pharmacology studies without the confounding factors of bronchoconstriction, mucus secretion, vascular effect or change in breathing that can indirectly stimulate airway nerves (Bergren 1997; Bonham et al. 1996; Kappagoda and Ravi 1989; Widdicombe 2003; Kollarik et al. 2003). As previously explained, PAR2 activation can result in different effects depending on the type of protease activating the receptor (Hollenberg et al. 2014). Although tryptase was considered as a relevant endogenous PAR2 agonist for eliciting a nerve reflex, it was not possible to test it due to cost issues. Trypsin was tested as

a surrogate, for the reason that both proteases cleave PAR2 at the same canonical site and reveal the same tethered ligand sequence SLIGRL on rodent and SLIGKV on human PAR2 receptors (Adams et al. 2011). As such, trypsin provoked the depolarisation of rat vagal nerves in a concentration-dependent manner, indicating that an endogenous PAR2 agonist can induce a signal in rat vagal nerves.

Because trypsin is not specific to PAR2 and can also stimulate PAR1 receptors that have also been found to activate airway nerves (Heuberger and Schuepbach 2019; Kwong et al. 2010), it was important to confirm these findings with selective PAR2 agonists. Two synthetic peptides mimicking the tethered ligand sequence revealed by tryptase cleavage, SLIGKV-NH2 and 2f-LIGRLO-NH2, were shown to be selective to PAR2 in the literature (Hollenberg et al. 1997; McGuire et al. 2004; Kawabata et al. 2004). These two peptides induced the depolarisation of rat isolated vagal nerves, albeit with a much smaller amplitude than trypsin, perhaps reflecting the involvement of PAR1 receptors in trypsin-induced depolarisation (Kwong et al. 2010). This difference in depolarisation could also be due to a difference in potency between the activation elicited by a protease and a mimetic peptide ligand (M. K. Yau, Liu, and Fairlie 2013).

To gain insights on whether non tryptase-like mechanisms of PAR2 activation could also depolarise rat vagal nerves, the small molecule AC55541 was tested as it has been suggested to be an allosteric PAR2 agonist, although this remains to be proven experimentally (J. N. Ma and Burstein 2013; Gardell et al. 2008). AC55541 also induced the depolarisation of rat vagal nerves, suggesting that non tryptase-like mechanisms of PAR2 activation could also stimulate rat vagal nerves.

Overall, 2f-LIGRLO-NH2 seemed to be the most potent of the PAR2 selective agonists tested to elicit rat vagus depolarisation, which is in accordance with the literature in the rat species (McGuire et al. 2004; M. K. Yau, Liu, and Fairlie 2013; Abey et al. 2006; Hollenberg et al. 2008). Although it is a peptide, its furoylated moiety confers increased stability to proteolytic degradation by aminopeptidases and is therefore amenable to *in vivo* studies (Kawabata et al. 2004; M. K. Yau, Liu, and Fairlie 2013). For these reasons, 2f-LIGRLO-NH2 was taken forward as the PAR2 agonist of choice, at the concentration eliciting submaximal depolarisation amongst the concentrations tested.

Further experiments confirmed that 2f-LIGRLO-NH2 was inducing a signal on vagal nerves via activation of PAR2 receptors. As such, the agonist induced the depolarisation of vagal nerves isolated from *Par2*$^{+/+}$ mice but not from littermate *Par2*$^{-/-}$ mice. As a control, vagal nerves from *Par2*$^{-/-}$ mice responded in the same way to TRPV4 and TRPV1 stimulation than *Par2*$^{+/+}$ mice, indicating that it was not due to a nonspecific effect of genetic disruption. To

confirm this finding in the rat species, the PAR2 antagonist P2pal-18S was tested against 2f-LIGRLO-NH2, at the same time validating this antagonist as an appropriate tool for blocking a PAR2-nerve signal.

Due to the fact that PAR2 are irreversibly activated by cleavage at different sites by different proteases and resulting in different signalling, developing PAR2 antagonists has proven to be difficult (Adams et al. 2011; Hollenberg et al. 2014). So far, small molecules have not been potent or shown to be biased agonists (M. S. Lee and Lerner 2019; M.-K. Yau et al. 2016; Suen et al. 2014). Commercially available PAR2 blocking antibodies such as SAM11 have also been used, however their selectivity has been questioned (Adams et al. 2012; M. K. Yau, Liu, and Fairlie 2013). In this thesis, P2pal-18S was chosen as a PAR2 antagonist because it was shown to be selective for PAR2 over PAR1 (Sevigny et al. 2011) and was developed as an allosteric antagonist. Therefore, it was hypothesised that it could theoretically inhibit PAR2 receptors independently from their mechanism of activation, although this has not been demonstrated experimentally (M. K. Yau, Liu, and Fairlie 2013; M. S. Lee and Lerner 2019; Sevigny et al. 2011; Covic et al. 2002).

P2pal-18S is a pepducin lipopeptide, meaning that it comprises a peptide sequence mimicking PAR2 intracellular loops and thereby providing receptor selectivity, and a palmitate lipid that renders the compound cell permeable. After translocation across the cell membrane, the pepducin binds to PAR2 intracellular domains and prevents interactions with G proteins (M. K. Yau, Liu, and Fairlie 2013; M. S. Lee and Lerner 2019). To control for non-specific protein interactions or cell permeation effects, a control Scrambled pepducin was custom-made for this thesis, containing the same aminoacids but assembled in a randomised sequence determined *in silico* as to not interact with PAR2 receptors. As compounds were made in limited quantities, it was not possible to perform a concentration-response and the single concentration of 10 µM was chosen, given that it was approximately 100 times its reported IC_{50} and was shown to block PAR2 signalling *in vitro* (Bagher et al. 2018; Lin et al. 2015; Sevigny et al. 2011). At this concentration, P2pal-18S inhibited the depolarisation induced by the PAR2 agonist 2f-LIGRLO-NH2 while the Scrambled peptide had no effect, further suggesting that PAR2 receptors activation depolarises rat vagal nerves and validating P2pal-18S as a suitable antagonist for blocking PAR2-nerve responses *in vitro*.

As explained in **2.2.1**, the vagus preparation does not specifically assess the effect of compounds on the terminals of airway sensory nerve fibres comprised within the vagus nerve and *in vitro* depolarisation is not synonym of *in vivo* nerve activation. Therefore, it was important to verify that PAR2 activation could in fact activate airway sensory nerve fibres, that is eliciting a depolarisation sufficient to provoke action potential firing. The single fibre

preparation was used in anaesthetised naïve rats, with both vagi disconnected from the CNS to prevent central reflexes. Because this thesis was interested in PAR2 as a TRPV4 activator and TRPV4 was shown to stimulate Aδ-fibres (Bonvini et al. 2016), only Aδ-fibres were studied. The PAR2 agonist 2f-LIGRLO-NH2 provoked a short-lasting increase in airway sensory Aδ-fibre firing at the maximum concentration tested (1 μM) and this signal was blocked by the PAR2 antagonist P2pal-18S. In addition, this increase in airway sensory fibre activity was not associated with a rise in intratracheal pressure, suggesting that it was due to a direct rather than indirect stimulation of airway nerves by airway smooth muscle contraction. These preliminary results seemed to indicate that stimulating PAR2 receptors can activate rat airway sensory Aδ-fibres. However, caution needs to be taken as only N=2 rats were used and the control Scrambled peptide could not be tested, for the reasons previously mentioned in **3.6** that performing this experiment required highly trained experts with limited availability.

The hypothesis that PAR2 was eliciting a nerve signal via activating TRPV4-P2X3 receptors was pursued using the rat vagus preparation that is more amenable to pharmacology experiments. Interestingly, both TRPV4 and P2X3-P2X2/3 antagonists GSK2193874 and AF-353 reduced PAR2 agonist 2f-LIGRLO-NH2-induced depolarisation, supporting the hypothesis that PAR2 activation depolarises rat vagal nerves via the TRPV4-P2X3 axis.

Before testing the role of PAR2 in the LAR, the ability of PAR2 receptors to provoke reflex and/or local bronchospasm was tested in naïve rats. Interestingly, the PAR2 agonist 2f-LIGRLO-NH2 did not seem to provoke a bronchospasm in anaesthetised and vagotomised rats in the single fibre preparation (N=2), suggesting that PAR2 activation did not cause local bronchospasm independently from a nerve reflex. This preliminary finding was confirmed *in vitro* as the PAR2 agonist 2f-LIGRLO-NH2 did not contract isolated tracheas nor small airways from naïve rats, in accordance with several studies showing that activation of PAR2 by SLIGRL-NH2 does not contract rat extra- or intrapulmonary airways (Chow, Moffatt, and Cocks 2000; Ricciardolo et al. 2000; Cocks et al. 1999). In conscious naïve rats, the PAR2 agonist 2f-LIGRLO-NH2 also did not provoke a Penh response. This finding confirmed that PAR2 activation does not provoke local bronchospasm but also suggested that it does not cause reflex bronchospasm in naïve rats. It is intriguing that PAR2 activation does not elicit small airways contraction while TRPV4 was suggested to do so (**5.4.4**), which contrasts with previous data suggesting that PAR2 signals through TRPV4 to stimulate airway nerves. This could however be due to a difference in PAR2 and TRPV4 receptors distribution between airway neurons and airway smooth muscle cells alongside the airway tree.

In the rat OVA model, the PAR2 antagonist P2pal-18S did not attenuate the LAR bronchospasm. However, the control Scrambled peptide seemed to inhibit the LAR. This could

not have been the result of mistakenly switching the P2pal-18S and Scrambled peptides, as the solutions were analysed and peptide sequences and biological activity proved to be correct. In addition, this study was repeated using new batches of compounds with confirmed sequences and bioactivity. Therefore, the effect of the Scrambled Peptide seemed to be a genuine effect of the compound, seemingly independent from targeting PAR2 receptors on rat vagal nerves, while the PAR2 antagonist P2pal-18S failed to show efficacy on the LAR. Given the half-life and wide distribution of P2pal-18S (Shearer et al. 2016), this lack of effect is unlikely due to pharmacokinetics considerations. In addition, the dose and route used (10 mg/kg, s.c.) seemed to inhibit airway sensory Aδ-fibre firing provoked by the PAR2 agonist 2f-LIGRLO-NH2 in the anaesthetised rat, although this needs confirmation (N=1).

Given these unexpected results, another PAR2 antagonist with a distinct mechanism of action was tested in the rat OVA model. The anti-PAR2 monoclonal antibody (mAb) MEDI2344 was used as it was demonstrated to possess a high affinity for PAR2 receptors and to reduce pain in rats. In contrast to P2pal-18S, this anti-PAR2 mAb binds to the extracellular N-terminus of PAR2 receptors and inhibits the proteolytic cleavage that unmasks the tethered ligand, thereby inhibiting subsequent signalling. However, the anti-PAR2 antibody MEDI2344 did not reduce the LAR in the rat OVA model. Therefore, these data were not in favour of a role for PAR2 receptors in driving the LAR in this model.

The small amplitude of the vagus signal elicited by PAR2 agonists and the absence of effect of two distinct PAR2 antagonists in the LAR may concur with the view that PAR2 receptors contribute to sensitise receptors such as TRPV4 on sensory nerves, rather than eliciting a nerve signal themselves (Mazzone and Undem 2016). Even in guinea pigs, the PAR2 agonists SLIGRL-NH2 or trypsin did not provoke cough but exaggerated cough to other stimuli (Gatti et al. 2006) whereas the TRPV4 agonist GSK1016790A was capable of provoking cough (Bonvini et al. 2016). So far, the effect of PAR2 receptors has been mostly studied on the activation of C-fibres and this remains controversial. In pain, only one group has reported that tryptase and SLIGRL-NH2 induce a calcium signal in rat sensory neurons isolated from dorsal root ganglia (DRG) (Steinhoff et al. 2000; Amadesi et al. 2004) while another group failed to show a current in mouse DRG neurons (Dai et al. 2004; 2007). In the airways, SLIGRL-NH2 did not appear to elicit significant calcium signal nor a current in capsaicin-sensitive rat vagal airway sensory neurons *in vitro* (Q. Gu and Lee 2006; 2009; 2010; Moss et al. 2015). *Ex vivo,* neither trypsin nor SLIGRL-NH2 were associated with capsaicin-sensitive C-fibre firing in guinea pigs and mice (Carr, Schechter, and Undem 2000; Kwong et al. 2010). Instead, studies showed that the PAR2 agonist SLIGRL-NH2 enhanced the excitability of rat vagal airway sensory neurons to other stimuli such as capsaicin *in vitro* and *in vivo* (Q. Gu and Lee 2006; 2009; 2010; Moss et al. 2015). Based on these data, PAR2 receptors are believed to sensitise

nociceptive neurons to other stimuli rather than being direct activators (Mazzone and Undem 2016). This has been a paradigm for many inflammatory mediators activating GPCRs on C-fibres, with the hypothesis that GPCR signalling needs to engage an ion channel such as TRP channels to generate nerve firing (Grace et al. 2013; Mazzone and Undem 2016), yet less data is available with Aδ-fibres.

It is also unclear how PAR2 induces TRPV4 signalling, this being likely due to the use of different compounds with variable degrees of selectivity and different cell types in the literature (Grant et al. 2007; Poole et al. 2013; Grace et al. 2014; Zhao et al. 2014; 2015; Cenac et al. 2015). It has been suggested that PAR2 activation opens TRPV4 channels via receptor operated gating, either by tyrosine kinase phosphorylation, PLA2-dependent generation of arachidonic acid metabolites or PKA-dependent pathways (Grace et al. 2014; Poole et al. 2013; Cenac et al. 2015; Zhao et al. 2014; 2015). On the other hand, studies have shown that PAR2 receptors sensitise TRPV4 channels via PLA2, PKA, PKC or PKD pathways (Grant et al. 2007; Poole et al. 2013). In this thesis, the experiments did not assess whether PAR2 agonists enhance the vagus response to TRPV4 stimulation and therefore no conclusion can be drawn on the sensitisation of TRPV4 by PAR2 receptors.

It is also unknown which cells expressing PAR2 would provoke a TRPV4-mediated nerve signal. PAR2 expression was scarcely found in murine capsaicin-sensitive vagal neurons by single-cell RT-PCR (Kwong et al. 2010) but this has not been investigated in capsaicin-insensitive neurons which may be those involved in TRPV4 signalling. It is possible that PAR2 and TRPV4 are expressed on the same non-neuronal cells surrounding airway neurons and interact via intracellular phosphorylation or secretion of secondary mediators. In pain, one study has suggested that a combination of inflammatory mediators known to trigger PKA or PKC pathways (5-HT, histamine, PGE_2, bradykinin, substance P) was engaging TRPV4 channels to provoke mechanical hyperalgesia, while each mediator tested alone did not activate TRPV4. The authors concluded that TRPV4 was activated when a subthreshold level of the secondary mediator intracellular cAMP was reached (Alessandri-Haber et al. 2006). Therefore, it could be speculated that TRPV4 channels are activated by a combination of several factors including PAR2, and that PAR2 blockade alone may not be sufficient to inhibit the involvement of TRPV4 in the LAR.

The difference between the effects of PAR2 and TRPV4 antagonists on the LAR may also reflect the fact that PAR2 receptors likely exert effects independently from TRPV4. As such, the PAR2 agonist 2f-LIGRLO-NH2 did not contract small airways contrary to the TRPV4 agonist. In fact, several studies have found that PAR2 activation with SLIGRL-NH2 provoked the relaxation of pre-contracted airways *in vitro* (Chow, Moffatt, and Cocks 2000; Ricciardolo

et al. 2000; Cocks et al. 1999) and even reduced the bronchospasm caused by 5-HT or histamine in rats and guinea pigs (Cocks et al. 1999; Cicala et al. 2001) or AHR to MCh in a mouse OVA model, with the authors concluding of a bronchoprotective role of PAR2 (De Campo and Henry 2005). Inhibiting this effect could therefore be deleterious in the LAR. Nonetheless, this bronchoprotective role has been contested, with several mouse OVA and HDM models showing a reduction in AHR upon PAR2 deletion or pharmacological blockade (Davidson et al. 2013; Asaduzzaman et al. 2018; 2015).

The PAR2 antagonist P2pal-18S and anti-PAR2 mAb MEDI2344 also did not inhibit the influx of eosinophils or neutrophils in rat airways after allergen challenge. In contrast, P2pal-18S has been associated with a reduction in eosinophilic inflammation in a mouse OVA model (Asaduzzaman et al. 2015) and PAR2 deletion or PAR2 blocking antibodies B5 and SAM11 reduced allergic airway inflammation in mouse OVA, HDM and cockroach models (Schmidlin et al. 2002; Davidson et al. 2013; de Boer et al. 2014; Asaduzzaman et al. 2015, 2018). However, the finding that PAR2 exerts pro-inflammatory effects has also been questioned, as the PAR2 agonist SLIGRL-NH2 inhibited allergic airway inflammation in a mouse OVA model (De Campo and Henry 2005).

Overall, this conflicted literature have led scientists to hypothesise that PAR2 receptors may be protective or detrimental depending on the stimuli present in the environment that activate or inactivate PAR2, likely depending on the animal model and pharmacological compounds used (M.-K. Yau et al. 2016; Nichols et al. 2012). In this thesis, only a tryptase-like mechanism of PAR2 activation was thoroughly studied on vagus nerve depolarisation, but other proteases released in the airways might have different effects or prevent this effect by disarming PAR2 receptors (Heuberger and Schuepbach 2019). P2pal-18S has been shown to inhibit trypsin-like induced calcium signalling but it did not seem to prevent PAR2-β-arrestin interactions *in vitro* and its effect on other PAR2 signalling pathways has not been characterised (Hollenberg et al. 2014). It remains unclear which PAR2 effects were inhibited or possibly enhanced by P2pal-18S and by the anti-PAR2 mAb MEDI2344 in the rat OVA model. Interestingly, the absence of effect of P2pal-18S or anti-PAR2 mAb contrasts with early studies showing a reduction in the LAR by tryptase inhibitors in human and animal studies (Krishna et al. 2001; Costanzo et al. 2003; Clark et al. 1995). This could however reflect the lack of selectivity of these early tryptase inhibitors (Pejler 2019). Testing more pathway-selective antagonists might provide new insights as to whether PAR2 receptors are involved in the LAR and which effects are beneficial or detrimental.

Nonetheless, a surprising and unexpected observation was the effect of the Scrambled peptide on the LAR. The Scrambled peptide was especially made for this thesis and no study

investigating P2pal-18S had so far reported the use of a control peptide. This control has been designed *in silico* with a sequence that should not bind to PAR2 receptors, however, its biological activity was not predicted against other targets. As the peptide is constituted of 18 aminoacids, it is highly possible that it interacts with unknown targets, especially as it is complexed to a palmitoyl moiety which renders it cell permeable and potentially increases its interaction with cell membrane proteins and distribution in the body (Covic et al. 2002; M. K. Yau, Liu, and Fairlie 2013). Using BLAST (Basic Local Alignment Search Tool) on the National Center for Biotechnology Information (NCBI) website, the Scrambled peptide sequence did not align with identified or known proteins involved in asthma pathophysiology. Interestingly, the reduction of the LAR was not associated with a reduction in airway inflammation, potentially indicative of an effect on the nervous system or bronchodilator properties. Therefore, these findings were extremely exciting as the Scrambled peptide may have potentially uncovered new targets in the LAR and asthma. With luck, this may be an example of drug discovery by serendipity. Thorough investigation is currently conducted to unravel the mechanism of action of this Scrambled peptide.

7 Summary and future directions

7.1 Summary of findings

When exposed to specific allergens, atopic asthmatics experience symptoms due to airflow limitation and despite available therapies, many patients remain uncontrolled. In the laboratory, inhalation of a high dose of allergen by atopic asthmatics recreates a prolonged episode of airway narrowing known as the LAR, associated with airway inflammation and AHR. An effect on the LAR harbours important predictive value of efficacy on airflow limitation in the clinic. Because the LAR and associated inflammation are reduced by corticosteroids, the LAR has been seen as the result of eosinophils and/or basophils recruitment, releasing mediators such as CysLTs causing bronchoconstriction, mucus secretion and oedema (Gauvreau, El-Gammal, and O'Byrne 2015). However, the failure of an anti-IL-5 therapy against the LAR has led to question the link between eosinophils and airflow limitation (Leckie et al. 2000) and not all patients respond to LTRAs (Szefler et al. 2005). Therefore, investigating alternative pathways driving the LAR could help to develop new therapies for atopic asthma.

The LAMA tiotropium has demonstrated efficacy on improving symptoms and baseline lung function in asthmatics, suggesting the existence of an enhanced cholinergic tone in asthma (Kerstjens et al. 2015; Peters et al. 2010; Paggiaro et al. 2016). While tiotropium has not been tested in allergen challenge studies in human, it reduced the LAR in rat and guinea pig OVA models (Raemdonck et al. 2012; Smit et al. 2014). The LAR was also blocked by anaesthesia in the rat OVA model, confirming the involvement of a central nerve reflex (Raemdonck et al. 2012). Therefore, the LAR could be due to allergen challenge resulting in the activation of airway sensory nerves and, after integration in the CNS, provoking a cholinergic reflex bronchospasm. The aim of this thesis was to investigate how airway sensory nerves could be activated and drive a cholinergic reflex in the LAR.

The TRPV4 channel activates airway sensory neurons in guinea pigs *in vivo* and depolarises human vagal nerves *in vitro* (Bonvini et al. 2016), showing translational potential. This effect seems to be indirect, with the release of ATP through Panx1 channels activating P2X3-P2X2/3 receptors on airway sensory neurons. This TRPV4-P2X3 axis has already been shown to provoke cough in guinea pigs, another airway reflex (Bonvini et al. 2016). Interestingly, ATP levels and PAR2 activating proteases, which are potential TRPV4 activators, are found upregulated in the airways of asthmatic patients (Idzko et al. 2007; Grant et al. 2007; Grace et al. 2014; Aubier et al. 2016; Sedgwick et al. 1991; S. E. Wenzel, Fowler, and Schwartz 1988). It was therefore hypothesised that TRPV4 could be one of the drivers of the LAR, by

activating airway sensory nerves via P2X3-P2X2/3 receptors and triggering an efferent cholinergic reflex.

To model the LAR, an OVA model set-up in Brown Norway rats was chosen for this thesis, as this model exhibits an EAR, LAR and airway eosinophilic inflammation responsive to gold standard therapies (Raemdonck et al. 2012; Hele et al. 2001). Importantly, this model was used to demonstrate the existence of a central cholinergic reflex in the LAR (Raemdonck et al. 2012).

Before testing this hypothesis, it was necessary to confirm whether the TRPV4-P2X3 axis can activate airway sensory nerves in the Brown Norway rat, as this has not been characterised before (Bonvini et al. 2016). At a concentration that elicited submaximal depolarisation of guinea pig vagal nerves, the synthetic TRPV4 agonist GSK1016790A depolarised rat isolated vagal nerves and this effect was inhibited by the TRPV4 antagonist GSK2193874. The depolarisation was also blocked by the P2X3-P2X2/3 antagonist AF-353 and Panx1 blocker probenecid, suggesting that TRPV4 activation depolarises rat vagal nerves via the release of ATP activating P2X3-P2X2/3 receptors, replicating findings from guinea pigs, mice and human vagal nerve preparations (Bonvini et al. 2016).

Because the vagus nerve contains fibres originating from other locations than the airways and that depolarisation is not synonym of nerve activation, the TRPV4 agonist was taken forward to more complex electrophysiology techniques. In a rat tracheal nerve preparation, the same concentration of TRPV4 agonist GSK1016790A elicited compound action potential firing of the RLN nerve attached to the trachea and this was blocked by the TRPV4 antagonist GSK2193874, suggesting that TRPV4 stimulation activates the terminals of airway fibres innervating rat large airways. In addition, TRPV4 activation does not contract rat trachea (McAlexander et al. 2014), suggesting that it directly activates airway neurons rather than an indirect activation caused by airway smooth muscle contraction. To study the *in vivo* translation of this effect and to confirm that TRPV4 can specifically activate airway sensory fibres, the single fibre preparation was used. In an anesthetised rat with both vagi disconnected from the CNS, aerosolising the TRPV4 agonist provoked a sustained action potential firing of an airway sensory Aδ-fibre, at a concentration that elicited submaximal Aδ-fibres firing in guinea pigs (Bonvini et al. 2016). It was not possible to test the P2X3 antagonist in this preparation, however the combination of these data suggested that TRPV4 activation can stimulate airway sensory nerves in the Brown Norway rat, via the release of ATP acting on P2X3-P2X2/3 receptors. This validated the use of Brown Norway rats for studying the TRPV4-P2X3 nerve axis in the LAR.

When investigated in the Brown Norway rat OVA model, both the TRPV4 antagonist GSK2193874 (300 mg/kg, i.p.) and P2X3-P2X2/3 antagonist AF-353 (30 mg/kg, i.p.) prevented the LAR. Although it is a high dose of TRPV4 antagonist, this dose was chosen for its inhibition of TRPV4-induced cough and airway sensory Aδ-fibre firing in guinea pigs (Bonvini et al. 2016). Furthermore, this dose did not inhibit capsaicin-induced cough nor capsaicin-induced airway sensory fibre firing in guinea pigs (Bonvini et al. 2016). Given that capsaicin is a TRPV1 agonist and that TRPV1 is the closest homolog of TRPV4, this suggested that the TRPV4 antagonist GSK2193874 was selective at this dose, although it was not tested against other TRP agonists. Overall, these findings supported the hypothesis that a TRPV4-P2X3 nerve axis could be involved in driving the LAR.

Interestingly, the TRPV4 antagonist GSK2193874 also significantly inhibited eosinophil and neutrophil influx in the airways at 24 h post OVA challenge. Because TRPV4 seems to exert its actions via the release of ATP, it is possible that TRPV4 activation stimulates other purinergic receptors such as P2X4 and P2X7 that have been involved in allergic airway inflammation (Idzko et al. 2007; Müller et al. 2011; Zech et al. 2016). However, the P2X3-P2X2/3 antagonist AF-353 also surprisingly reduced airway inflammation in the rat OVA model. In the literature, P2X3 expression seems restricted to sensory neurons at baseline (Burnstock 2007). Therefore, these data could suggest that airway nerves actively contribute to driving airway inflammation as a positive feedback loop. In line with this hypothesis, the LAMA tiotropium demonstrated a signal on reducing exacerbations in asthmatic patients, although with a mild effect (Vrijlandt et al. 2018; Kerstjens et al. 2012).

Overall, these first chapters suggested that a TRPV4 agonist seemed to stimulate airway sensory nerves in naïve rats via the ATP-P2X2/3-P2X3 axis and that both a TRPV4 and P2X3-P2X2/3 antagonist reduced the LAR in the rat OVA model. These data made a strong case for a role of the TRPV4-P2X3 axis in activating afferent nerves and triggering a cholinergic reflex bronchospasm in the LAR. However, these experiments did not ascertain whether it was involved in the LAR via triggering a cholinergic reflex. In addition, the separate blockade of TRPV4 and P2X3-P2X2/3 did not ascertain the link between TRPV4 and P2X3-P2X2/3 receptors in the LAR. To further test this hypothesis, a pharmacological approach was conducted in naïve rats, with the aim of testing whether activation of the TRPV4-P2X3 axis could provoke LAR-like responses in naïve rats. Aerosolising the TRPV4 agonist GSK1016790A (10 µg/ml) elicited an increase in Penh in conscious naïve rats, an effect inhibited by the TRPV4 antagonist GSK2193874. However, this increase in Penh was not blocked by the LAMA tiotropium nor by the P2X3-P2X2/3 antagonist AF-353, suggesting that airway sensory nerves firing induced by the TRPV4-P2X3 axis does not transduce into a cholinergic reflex in naïve rats. However, this may not necessarily be the case in the rat OVA

model, as several studies have highlighted that allergen-induced inflammation enhances airway sensory nerves activity and is associated with more permissive central and efferent synapse transmissions (Undem and Taylor-Clark 2014). It is therefore possible that an afferent TRPV4-P2X3-induced signal could trigger an efferent reflex in presence of airway inflammation in the rat OVA model but not in naïve rats.

Nonetheless, TRPV4 activation did provoke an increase in Penh in naïve rats, suggesting that TRPV4 provoked local bronchospasm independently from a nerve reflex. This finding was unexpected in the rat species, given that TRPV4 agonists were not shown to contract rat trachea (McAlexander et al. 2014). In light of these unexpected results, it was necessary to confirm if this increase in Penh was indicative of a bronchospasm rather than nose obstruction or alteration in breathing. Aerosolising the TRPV4 agonist GSK1016790A (100 ng/ml) provoked a short-lasting increase in intratracheal pressure in an anaesthetised and vagotomised rat, suggesting that TRPV4 activation can indeed provoke local bronchospasm independently from a nerve reflex. Further *in vitro* studies on airways isolated from naïve rats investigated the mechanism where, as expected, the TRPV4 agonist GSK1016790A did not contract isolated rat tracheas, confirming findings from the literature (McAlexander et al. 2014). In contrast, it provoked the contraction of rat small airways, demonstrated using PCLS, an effect blocked by the TRPV4 antagonist GSK2193874. This contraction was also inhibited by the Panx1 blocker probenecid, suggesting the involvement of ATP release. In addition, TRPV4-induced contraction was not inhibited by the P2X3-P2X2/3 antagonist AF-353 confirming *in vivo* Penh findings, but was inhibited by the P2X4 antagonist 5-BDBD, implying that TRPV4 activation contracted naïve rat small airways via the ATP-P2X4 axis.

In human and guinea pig large airways, TRPV4 activation induces airway smooth muscle contraction by stimulating P2X4 receptors on mast cells and provoking the release of CysLTs. It is unlikely that TRPV4-induced rat small airways contraction involves mast cells, given that rat mast cells are preferentially located in large airways (Miller and Pemberton 2002) and rat trachea does not contract to TRPV4 agonists (McAlexander et al. 2014). Accordingly, TRPV4-induced Penh was not blocked by a combination of antagonists targeting receptors of the mast cell mediators 5-HT, histamine and LTD_4 in conscious naïve rats. The relevance of this rat small airways contraction is uncertain given the disparities in airway smooth muscle and mast cell biology between rat and human (Zosky and Sly 2007; Stevenson and Belvisi 2008; Aun et al. 2018). Regarding the LAR, the relevance of this finding is also unknown. It is plausible that TRPV4 induces both a P2X3-mediated reflex bronchospasm and a P2X4-mediated local bronchospasm, depending on the location of $TRPV4^+$ cells and types of P2X receptors expressed in the vicinity. It is however unclear whether the TRPV4-P2X4 axis would contribute meaningfully to the LAR compared to the TRPV4-P2X3 axis in the rat OVA model and this

could not be tested due to a lack of selective P2X4 antagonists suitable for *in vivo* use. Interestingly, the contraction induced by the TRPV4-P2X4 axis was observed in naïve rat and guinea pig tissues as well as in control human airway tissue, in an allergen and IgE-independent manner (Bonvini et al. 2020). Therefore, the TRPV4-P2X4 axis may be more relevant in provoking nonspecific bronchospasm, including in nonatopic asthma.

So far, presented data suggested that TRPV4 and P2X3-P2X2/3 receptors were involved in driving the LAR in the rat OVA model. However, it remained unknown how allergen challenge would result in the activation of the TRPV4-P2X3 axis, that would trigger a delayed and prolonged bronchospasm characteristic of the LAR. Therefore, the final aim of this thesis was to investigate potential activators of the TRPV4-P2X3 axis in the LAR. It has been hypothesised that mediators released by mast cells during the EAR could initiate a cascade of events leading to the LAR (Bradding and Arthur 2016). Among these mediators, the PAR2 activating protease tryptase is found elevated in the airways of atopic patients after allergen challenge (Sedgwick et al. 1991; S. E. Wenzel, Fowler, and Schwartz 1988) and a tryptase inhibitor reduced the LAR but not the EAR in atopic asthmatics (Krishna et al. 2001). Interestingly, studies have shown that PAR2 activation induces a signal in spinal sensory neurons that transduces into an efferent pain response by activating and/or sensitising TRPV4 channels (Grant et al. 2007; Poole et al. 2013; Sipe et al. 2008; Cenac et al. 2015). Therefore, it was hypothesised that PAR2 receptors could be promising candidates for activating a TRPV4-P2X3 nerve reflex in the LAR.

The first step consisted in testing the effect of PAR2 activation on airway sensory nerves in naive rats. The PAR2 agonist 2f-LIGRLO-NH2 induced the depolarisation of rat isolated vagal nerves, an effect blocked by the PAR2 antagonist P2pal-18S but not by the Scrambled version of the antagonist. This depolarisation was also inhibited by the TRPV4 antagonist GSK2193874 and P2X3-P2X2/3 antagonist AF-353. Furthermore, the PAR2 agonist induced airway sensory Aδ-fibre firing in anaesthetised rats, a signal inhibited by the PAR2 antagonist P2pal-18S. Overall, these preliminary data implied that stimulating PAR2 receptors can activate rat airway sensory nerves via the TRPV4-P2X3 axis. Curiously, the PAR2 agonist did not seem to provoke local bronchospasm contrary to TRPV4 stimulation.

When tested in the rat OVA model, the PAR2 antagonist P2pal-18S did not prevent the LAR nor did it reduce airway inflammation. A second approach was used to block PAR2 receptors, however the anti-PAR2 monoclonal antibody MEDI2344 also failed to reduce the LAR and associated airway inflammation. These findings do not support the hypothesis that PAR2 receptors are involved in the LAR in this model. Importantly, these findings also do not seem to support the hypothesis that PAR2 receptors are activators of a TRPV4-P2X3 reflex in the

LAR. Given the polymodal nature of TRPV4, it is however possible that TRPV4 is activated by a combination of inflammatory mediators including PAR2 (Alessandri-Haber et al. 2006) and that blockade of a single mediator is not effective in inhibiting TRPV4-effects in the LAR. In fact, many distinct physical and chemical modalities such as hypotonicity, shear stress or arachidonic acid metabolites have been reported to stimulate TRPV4 and it is still unclear whether these stimuli or endogenous mediators directly affect the channel. In addition, inflammatory mediators such as histamine or 5-HT have also been reported to indirectly stimulate TRPV4 channels via protein kinases or tyrosine kinases phosphorylation (White et al. 2016). Therefore, further studies are needed to investigate other activators of TRPV4 in the LAR, to better understand how TRPV4 channels can be activated following allergen challenge.

More surprisingly, the control Scrambled peptide inhibited the LAR. Thorough validation work confirmed that it was not due to compounds being switched. This Scrambled peptide sequence was constructed *in silico* with the requirement of not binding to PAR2 receptors, but given its 18-aminoacids length, it is highly possible that it binds to other proteins or lipid compounds in the body. As the Scrambled peptide did not significantly reduce airway inflammation associated with the LAR, this compound may have bronchodilator properties. Studying its mechanism of action holds promise to identify new targets effective in reducing airflow limitation in atopic asthma.

Overall, the main findings from this thesis suggested that activation of the TRPV4-P2X3 axis stimulates airway sensory nerves in naïve rats and that TRPV4 and P2X3-P2X2/3 receptors are involved in driving the LAR in the rat OVA model, supporting this thesis hypothesis that a TRPV4-P2X3 nerve reflex could drive the LAR. However, because TRPV4 activation did not seem to provoke a P2X3-cholinergic bronchospasm in naïve rats, further studies are needed to confirm this hypothesis. TRPV4-P2X3 nerve activation may elicit a reflex bronchospasm in the context of allergic airway inflammation but not in the naïve state.

Further sections will outline the limits of this work and possible future studies to continue this project, along with the potential clinical relevance of these findings.

7.2 Thesis limitations

There are a number of limitations to the methods used within this thesis, some of which have been previously outlined. This section summarises the main limitations.

Airway sensory nerve activation was mostly studied using the isolated vagus nerve preparation, as it allows relatively high throughput pharmacology studies with a reduced number of animals. However, this technique does not specifically evaluate the effect of compounds on the terminals of airway sensory fibres present within the vagus nerve, nor is depolarisation synonym of action potential firing. Where possible, this technique was complemented by the tracheal nerve and single fibre preparations but only a limited number of experiments could be performed due to the limited availability of the high-skilled operators running these labour-intensive techniques.

The choice of an OVA-driven model has been highly criticised in the asthma field, for the main reasons that OVA is not a clinically relevant allergen and that systemic sensitisation with an adjuvant and the development of tolerance to repeated OVA challenges does not reflect the course of asthma disease (Stevenson and Birrell 2011). However, it was not possible to use an alternative since the LAR has not been successfully modelled using more clinically relevant allergens such as HDM.

The use of Penh measured by whole body plethysmography to assess bronchospasm has also been controverted, for the main reason that Penh is not a direct measure of airway resistance and can be influenced by upper airways obstruction, alteration in breathing and environmental factors (Lundblad et al. 2007; J. Bates et al. 2004; J. H. T. Bates and Irvin 2003). Because this thesis investigated airway reflexes and that anaesthesia abolished the LAR in the rat OVA model, it was not possible to measure airway resistance (Raemdonck et al. 2012). In addition, it was also not ethically acceptable to restrain animals for the duration of the LAR, ruling out alternative techniques such as double-chamber plethysmography.

The choice of the rat species to investigate airway reflexes and bronchospasm can also be criticised. Indeed, the rat species exhibits several differences in airway innervation compared to human. The rat airway smooth muscle is also less responsive to bronchoconstrictors, with different mediators released from mast cells compared to human (Stevenson and Belvisi 2008; Canning and Chou 2008). In this thesis, the TRPV4-P2X3 axis seemed to depolarise rat vagal nerves in the same way than human vagal nerves (Bonvini et al. 2016), suggesting translation of the afferent signal. However, efferent reflexes seem to differ between rat and human. As such, rats do not present evidence of a cough reflex. In contrast, guinea pigs exhibit a cough reflex and have been the preferred species for studying airway reflexes (Stevenson and Belvisi

2008; Canning and Chou 2008). However, guinea pigs are also very sensitive to nonspecific AHR, with baseline levels of eosinophil-like cells in their airways. In addition, guinea pig models of the LAR have been inconsistent (Stevenson and Belvisi 2008). Therefore, the rat species was preferred for studying allergic specific mechanisms of the LAR in this thesis.

Another important limit of this work is the use of a single TRPV4 antagonist GSK2193874 and P2X3-P2X2/3 antagonist AF-353 to study the involvement of TRPV4 and P2X3-P2X2/3 receptors in the LAR in the rat OVA model. In addition, both antagonists were only used at a single dose in the rat OVA model. It is therefore possible that other receptors were targeted by these antagonists and were responsible for the observed effects. This is also true for each antagonist used *in vitro* in this thesis. This will be further discussed in the next section, along with possible future studies to circumvent these caveats.

7.3 Future directions

7.3.1 Validate the role of TRPV4 in the LAR

The assumption that TRPV4 is involved in the LAR is based on the testing of a single dose (300 mg/kg, i.p.) of the TRPV4 antagonist GSK2193874 in the rat OVA model. While this antagonist has proven selective *in vivo* against TRPV1-induced airway fibre firing in guinea pigs (Bonvini et al. 2016) and *in vitro* against TRPV1-induced depolarisation of rat vagal nerves in this thesis, it is possible that it targeted other receptors in the rat OVA model. It is therefore necessary to confirm these findings, potentially by performing a concentration-response of the TRPV4 antagonist GSK2193874 and by testing structurally distinct TRPV4 antagonists in the rat OVA model.

It is intriguing that a TRPA1 antagonist prevented the LAR in the same rat OVA model (Raemdonck et al. 2012). TRPA1 characteristically activates airway C-fibres (Adcock et al. 2014), while TRPV4 seems to preferentially activate Aδ-fibres (Bonvini et al. 2016). Studies have suggested that activation of C-fibres can enhance Aδ-fibres induced signal (Kesler and Canning 1999; Canning, Reynolds, and Mazzone 2001; Mazzone and Canning 2002b). It is possible that TRPA1 and TRPV4 pathways interact to provoke an efferent reflex in the LAR. To test the interaction of TRPA1 and TRPV4 pathways on airway sensory nerves, TRPV4 and P2X3 antagonists could be tested against TRPA1-induced nerve signal and vice-versa, *in vitro* on isolated vagal nerves and *in vivo* on airway sensory fibre firing. As TRPV4 and TRPA1 are potentially stimulated by different endogenous and exogenous compounds, studying this crosstalk might provide information on how different stimuli could lead to similar symptoms due to airflow limitation.

Importantly, these findings could however suggest an off-target effect of the TRPV4 antagonist GSK2193874 on TRPA1 receptors. *In vitro*, GSK2193874 demonstrated an IC_{50} of >25000 nM for TRPA1 compared to 40 nM for TRPV4 in human cell assays, suggesting that it is not due to an off-target effect (Thorneloe et al. 2012) but this may not relate to *in vivo* exposure with GSK2193874 (300 mg/kg, i.p.). This emphasizes the importance of validating the role of TRPV4 in the LAR using distinct TRPV4 antagonists such as the clinical compound GSK2798745 and/or performing a concentration-response of GSK2193874.

7.3.2 Determine whether P2X3 or P2X2/3 receptors are involved in TRPV4-induced nerve signal and the LAR

As for the TRPV4 antagonist GSK2193874, the assumption that P2X3-P3X2/3 receptors mediate TRPV4-induced vagus depolarisation and are involved in the LAR in the rat OVA model is based on a single antagonist AF-353, at a single dose *in vitro* and *in vivo*. In the literature, AF-353 has proven selective to recombinant human P2X3-P2X2/3 receptors against P2X1, P2X2, P2X4, P2X5, and P2X7 in cell lines at the concentration used *in vitro* (10 µM) (Gever et al. 2010). However, this has not been tested in the rat species nor *in vivo*. It is therefore necessary to confirm these findings, potentially by performing a concentration-response of the P2X3-P2X2/3 antagonist AF-353 and by testing distinct antagonists in the rat OVA model.

Furthermore, additional studies could determine whether P2X2/3 heterotrimers and/or P2X3 homomers are involved in TRPV4-induced nerve signalling and the LAR. Circumstantial evidence obtained in guinea pigs suggest that TRPV4 stimulates airway sensory neurons via the release of ATP that preferentially stimulates P2X2/3 heterotrimers compared to P2X3 homomers (Bonvini et al. 2016), however this has not been confirmed. New compounds such as BLU-5937, BAY1817080 and S-600918 that exhibit greater selectivity for P2X3 compared to P2X2/3 receptors could be tested against TRPV4-induced nerve signal and against the LAR in the rat OVA model.

7.3.3 Investigate if the TRPV4-P2X3 axis triggers reflex bronchospasm in presence of allergic airway inflammation

The TRPV4-P2X3 axis stimulated rat vagal nerves and the TRPV4 and P2X3-P2X2/3 antagonists prevented the LAR in the rat OVA model, suggesting that activation of the TRPV4-P2X3 afferent axis triggers a cholinergic reflex driving the LAR. However, a TRPV4 agonist did not seem to provoke a P2X3-mediated cholinergic reflex in naïve rats. It was hypothesised that a TRPV4-P2X3 afferent nerve signal may trigger an efferent cholinergic reflex in the presence of allergic airway inflammation but not in naïve rats. This notion of a disease-specific effect would be particularly advantageous for an asthma therapy and merits further investigation.

Firstly, it is important to confirm that TRPV4 stimulation activates airway sensory nerves in Brown Norway rats by activating P2X3-P2X2/3 receptors. In this thesis, this was mostly investigated using the isolated vagus preparation and only N=1 experiment assessed the effect of the TRPV4 agonist on Aδ-fibre firing. This single fibre experiment needs to be repeated and TRPV4 agonist-induced Aδ-fibre firing tested against TRPV4 and P2X3-P2X2/3

antagonists. It would also be interesting to test if the direct activation of P2X3-P2X2/3 receptors by αβ-MeATP can provoke a cholinergic bronchospasm in naïve rats.

If it is confirmed that TRPV4 elicits Aδ-fibre firing via P2X3-P2X2/3 receptors, further studies could investigate whether TRPV4 activation elicits a cholinergic reflex in the presence of allergic airway inflammation but not in the naïve state.

A small in-house study found that TRPV4-induced Penh response was not different between OVA-sensitised and SAL-sensitised rats, in the absence of any OVA challenge (**Figure 7-1**), suggesting that sensitisation does not influence TRPV4-P2X3 efferent output. In addition, the magnitude of the depolarisation induced by the TRPV4 agonist GSK1016790A and P2X3 agonist αβ-MeATP were not statistically different between OVA-sensitised and SAL-sensitised rats, suggesting that sensitisation does not upregulate TRPV4-P2X3 vagal nerve signalling (**Figure 7-2**). These results were expected as systemic sensitisation to OVA does not provoke airway inflammation itself. It is hypothesised that airway inflammation triggered by OVA challenge in OVA-sensitised animals would be required for the TRPV4-P2X3 axis to trigger reflex bronchospasm.

To test this hypothesis, the effect of aerosolising the TRPV4 agonist GSK1016790A could be tested in conscious OVA-sensitised rats 24 h after OVA-challenge compared to SAL-challenge, timepoint at which rats have recovered from the LAR but still exhibit significant airway inflammation (S. L. Underwood et al. 2002). The LAMA tiotropium and P2X3-P2X2/3 antagonist AF-353 could be tested against this TRPV4-induced bronchospasm, to attest if it involves a P2X3-cholinergic reflex in presence of OVA-induced inflammation contrary to the naïve state.

In a pilot study, isolated vagus responses were compared one day after OVA challenge in SAL versus OVA-sensitised rats. Although preliminary, the magnitude of the depolarisation induced by the TRPV4 agonist GSK1016790A and P2X3 agonist αβ-MeATP did not appear different between OVA-sensitised and SAL-sensitised rats (N=2), which could imply that airway inflammation does not upregulate TRPV4-P2X3 signalling on the vagus nerve itself (**Figure 7-3**). Nonetheless, as for previously presented data, isolated vagal trunks may not reflect a local upregulation of P2X3-P2X2/3 receptors on airway sensory terminals nor of TRPV4 on surrounding cells.

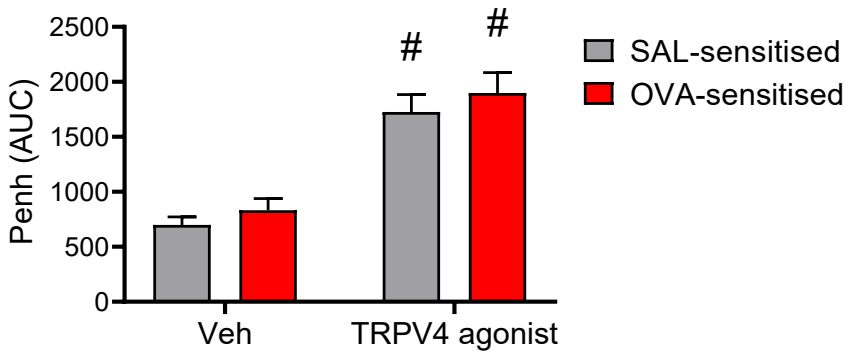


Figure 7-1 Effect of OVA sensitisation on TRPV4-induced Penh response

Male Brown Norway rats were sensitised with OVA or the vehicle SAL (Alum^{TM} in saline) on day D0, D14 and D21. On day D28, conscious rats were challenged with the vehicle (1% EtOH), followed by the TRPV4 agonist GSK1016790A (10 μg/ml). Drugs were aerosolised for 10 min and Penh recorded for 20 min using FWBP. N=8/group. Wilcoxon-test $^{\#}p<0.05$ comparing TRPV4 agonist response to its paired vehicle. Mann-Witney test non-significant comparing TRPV4 agonist responses between SAL and OVA-sensitised rats.

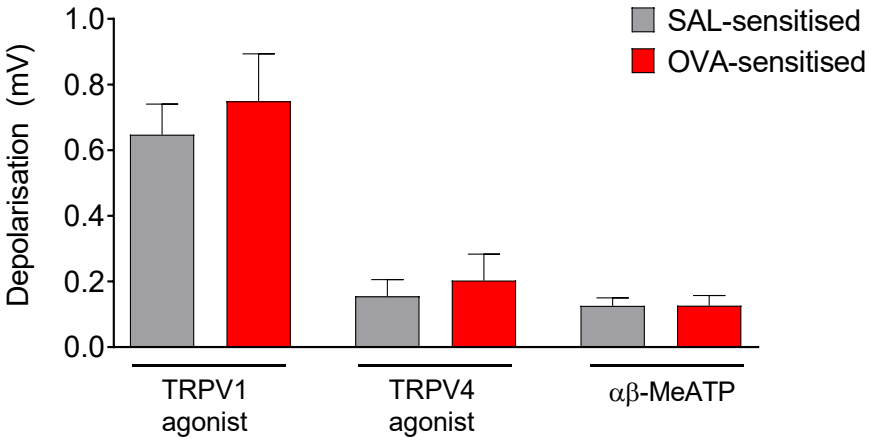


Figure 7-2 Effect of OVA sensitisation on TRPV4-induced vagus depolarisation

Male Brown Norway rats were sensitised with OVA or the vehicle SAL (Alum^{TM} in saline) on day D0, D14 and D21. On day D28, rats were euthanised and their vagal nerves isolated and successively stimulated with the TRPV4 agonist GSK1016790A (300 nM), αβ-MeATP (100 μM) and reference stimulus TRPV1 agonist capsaicin (1 μM) and the depolarisation of the nerve assessed. Data expressed as mean ± S.E.M., N=4 rats, Mann-Whitney test non-significant between SAL and OVA-sensitised rats.

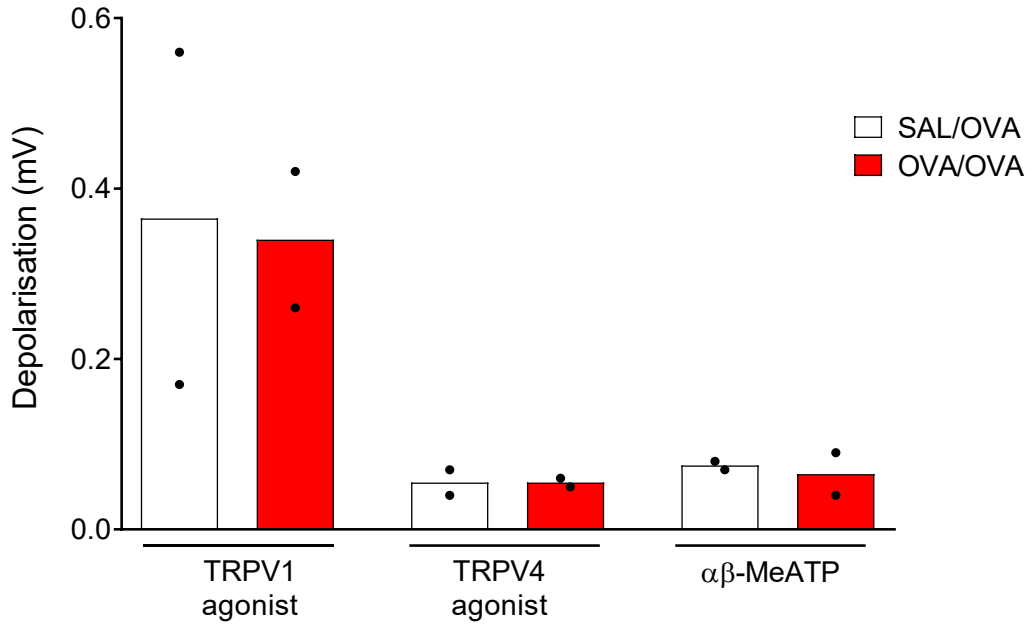


Figure 7-3 Effect of OVA challenge on TRPV4-induced vagus depolarisation

Male Brown Norway rats were sensitised with OVA or the vehicle SAL (Alum^{TM} in saline) on day D0, D14 and D21. On day D28, rats were challenged with OVA aerosol. One day after challenge, rats were euthanised and their vagal nerves isolated and successively stimulated with the TRPV4 agonist GSK1016790A (300 nM), αβ-MeATP (100 µM) and reference stimulus TRPV1 agonist capsaicin (1 µM) and the depolarisation of the nerve assessed. N=2 rats.

7.3.4 Investigate the role of TRPV4 and P2X3 in allergic airway inflammation

Both the TRPV4 and P2X3-P2X2/3 antagonists reduced cellular inflammation in the rat OVA model. This observation could corroborate the paradigm that eosinophilic recruitment drives the LAR. In this model, previous studies have suggested that eosinophilic recruitment peaks later than the LAR (S. L. Underwood et al. 2002) and repeated OVA challenges were associated with reduced eosinophilic inflammation but a similar magnitude of the LAR after each challenge (Flajolet et al. 2018), suggesting that airway eosinophilia does not drive the LAR in this model. However, the inflammatory events driving the LAR in this model have not been extensively characterised. It would be important to investigate if airway eosinophilia drives the LAR in this model, for instance by using an anti-IL-5 antibody, to validate the relevance of the model compared to human.

The fact that P2X3-P2X2/3 blockade attenuated airway eosinophilia could suggest that airway nerves contribute to the development of airway inflammation as a positive feedback loop. P2X3 receptors have been mostly studied in the context of neuropathic pain and cough, but their role in airway inflammation has received less attention. Their role in driving allergic airway inflammation could be studied in mouse models using relevant allergens such as HDM, using *P2x3*$^{-/-}$ mice and the P2X3-P2X2/3 antagonist AF-353. At the difference of investigating the contribution of airway nerves using LAMAs such as tiotropium, studying P2X3-P2X2/3 receptors might be more specific and indicative of a role for airway nerves in allergic airway inflammation. The role of P2X3-P2X2/3 could also be studied on AHR in these models.

Aside from a nerve reflex mediated by P2X3-P2X2/3 receptors, TRPV4 could also participate in driving allergic airway inflammation by releasing ATP activating purinergic receptors such as P2X1, P2X4 and P2X7 on immune cells (Idzko et al. 2007; Müller et al. 2011; Zech et al. 2016; Junger 2011). So far, the role of TRPV4 in driving allergic airway inflammation remains elusive, with only one published report (Palaniyandi et al. 2019). This could be studied in mouse HDM models using *Trpv4*$^{-/-}$ mice and the TRPV4 antagonist GSK2193874. The role of TRPV4 could also be studied on the development of AHR in these models. Depending on the outcome, further studies could decipher on which cells TRPV4 is important for driving allergic airway inflammation. To determine if TRPV4 is needed on hematopoietic cells or structural cells, bone marrow chimeric mice could be used. Further studies could investigate if TRPV4 is needed on T cells to drive inflammation, by deleting TRPV4 receptors on CD4^{+} T cells and performing adoptive transfers of wild-type or *Trpv4*$^{-/-}$ HDM-specific CD4^{+} T cells in a mouse HDM model. Translational data could be obtained by studying the expression and functionality of TRPV4 channels on human immune cells.

7.3.5 Investigate the role of TRPV4 in efferent parasympathetic nerves

TRPV4 activation may also play a role in the activation of efferent cholinergic nerves, their release of ACh or in the modulation of muscarinic receptors response. Rat paratracheal ganglia neurons were found to be immunoreactive for P2X2 and to respond to ATP (B. Ma et al. 2005), therefore a TRPV4-P2X2 axis could be involved in modulating parasympathetic output. This could be tested using an organ bath preparation of isolated rat and human tracheas, which contain parasympathetic ganglia in the vicinity of the smooth muscle. Electrical field stimulation (EFS) of these tracheas induces the release of endogenous ACh contained in cholinergic nerve terminals, triggering airway smooth muscle contraction. The TRPV4 antagonist could be tested against EFS-induced contraction to determine if TRPV4 is needed in this cholinergic pathway. To test if TRPV4 is involved in enhancing this cholinergic pathway, the TRPV4 agonist could be tested on EFS-induced contraction. These responses could be compared to exogenous ACh, to test if TRPV4 modulates the response to ACh on muscarinic receptors. A preliminary experiment found no effect of the TRPV4 antagonist GSK2193874 on EFS-induced contraction (N=1) (**Figure 7-4**), which may suggest that TRPV4 is not involved in the release of ACh from cholinergic nerves and/or muscarinic receptors signalling.

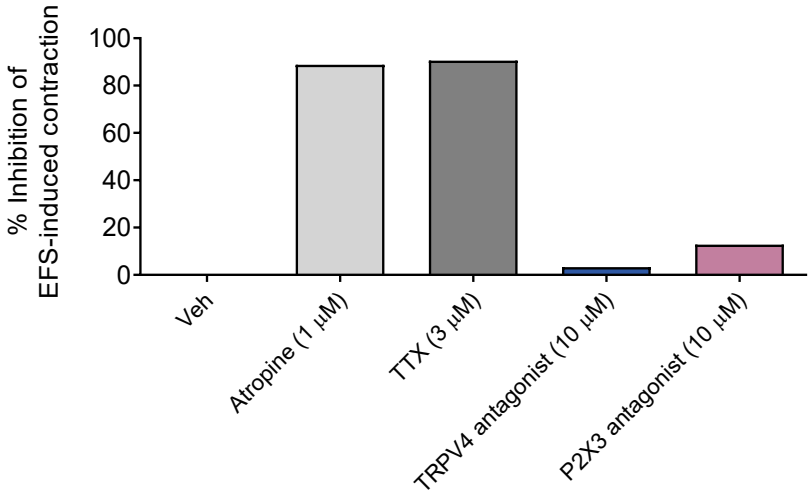


Figure 7-4 Effect of the TRPV4 antagonist on EFS-induced tracheal contraction

Tracheal segments were isolated from a naïve Brown Norway rat (N=1) and incubated in an organ bath. Electrical Field Stimulation (EFS) was applied to stimulate the release of ACh from cholinergic nerves (EFS: 40 V, 0.5 msec, 32 Hz for 15 sec). Tissues were incubated for 30 min with a single antagonist. The graph represents the percentage inhibition of EFS-induced contraction for each antagonist. TRPV4 antagonist: GSK2193874; P2X3 antagonist: AF-353; Atropine: non-selective muscarinic antagonist, positive control that ACh release induces the contraction; TTX: Tetrodotoxin non-selective $Na_V1.1$-9 blocker, positive control that nerve activation induces the contraction.

7.3.6 Investigate the role of TRPV4 and P2X4 in local bronchospasm

TRPV4 has been shown to contract human and guinea pig large airways via activating P2X4 receptors and inducing the release of CysLTs from mast cells, independently from a nerve reflex (Bonvini et al. 2020). In this thesis, TRPV4 induced rat small airways contraction also via activating P2X4 receptors, but it seemed to involve a different mechanism than mast cell mediators release. It would be interesting to study if TRPV4 activation can also contract human small airways independently from mast cells, using human PCLS. This could reflect another mechanism by which TRPV4 could provoke local bronchospasm. A CysLTs-independent mechanism would be particularly interesting in the clinic given that an important number patients do not respond to LTRAs (Szefler et al. 2005).

7.3.7 Validate the role of PAR2 in the LAR and investigate other activators of the TRPV4-P2X3 axis

While results from this thesis suggested that PAR2 activation induces a TRPV4-P2X3 vagal nerve signal *in vitro*, the PAR2 antagonist P2pal-18S and anti-PAR2 antibody MEDI2344 did not inhibit the LAR in the rat OVA model. However, it is unclear which PAR2 signalling pathways were blocked by these two antagonists and because of PAR2 biased signalling, distinct PAR2 pathways may lead to deleterious or beneficial effects. Therefore, testing distinct antagonists that specifically target certain PAR2 signalling pathways could provide more information on the role of PAR2 in the LAR.

Nevertheless, data from this thesis did not favour PAR2 receptors as important activators of the TRPV4-P2X3 axis in the rat OVA model. Therefore, further studies are needed to investigate how allergen challenge could lead to the delayed and prolonged activation of the TRPV4-P2X3 axis leading to the LAR.

7.3.8 Investigate the mechanism of action of the Scrambled peptide

A surprising finding was the efficacy of the Scrambled peptide on the LAR in the rat OVA model. This Scrambled peptide was designed as a randomised sequence of the PAR2 antagonist P2pal-18S and *in vitro* bioassays suggested that it does not block PAR2-induced nerve signalling. However, this compound was not tested *in vivo* against PAR2-nerve signal. It is therefore important to confirm that it does not inhibit PAR2 agonist-induced airway sensory Aδ-fibre firing in anaesthetised rats.

Future studies could investigate the mechanism of action of the Scrambled peptide, as it may help to discover new targets for reducing airflow limitation in asthma. The fact that it did not significantly reduce airway inflammation could reflect bronchodilator properties. This could be

tested by performing a concentration-response of the Scrambled peptide on rat and human pre-contracted isolated airways using the organ bath preparation. To decipher potential targets, the Scrambled peptide activity could be screened against a library of receptors *in vitro*.

7.3.9 Clinical studies and perspective

This thesis was based on the hypothesis that airway nerves play an important role in triggering the LAR, ultimately with the aim to decipher new mechanisms leading to airflow limitation in atopic asthma. The observation that inhibiting TRPV4 and P2X3-P2X2/3 receptors prevented the LAR in the rat OVA model, in association with the findings that anaesthesia and a LAMA blocked this reaction (Raemdonck et al. 2012), indeed suggests that airway nerves could play a role in driving the LAR. To investigate if this translates to human, TRPV4 and P2X3-P2X2/3 antagonists as well as LAMAs could be tested in allergen challenge studies in atopic asthmatics. TRPV4 and P2X3-P2X2/3 antagonists have already been developed for clinical use (Abdulqawi et al. 2015; Ludbrook et al. 2019) and, while the commercially available tiotropium has proven effective on baseline lung function in asthmatic patients (Kerstjens et al. 2015; Peters et al. 2010; Paggiaro et al. 2016), it has never been tested in allergen challenge studies.

Testing a P2X3-P2X2/3 antagonist in allergen challenge studies could perhaps be a more specific indication that airway nerves are involved in asthma pathophysiology than a LAMA or TRPV4 antagonist, given that ACh is released by non-neuronal sources and TRPV4 exerts its actions via ATP release. In fact, targeting P2X3-P2X2/3 receptors seems particularly promising for inhibiting airway reflexes in human, as the P2X3-P2X2/3 antagonist AF-219 was associated with a 75% reduction in refractory chronic cough in a proof-of-concept study (Abdulqawi et al. 2015). Testing a P2X3-P2X2/3 antagonist could also give the opportunity to test the contribution of airway nerves in driving inflammation in asthma. Given the blockade of airway inflammation by the P2X3-P2X2/3 antagonist in the rat OVA model, targeting airway nerves may have additional effects than solely reducing symptoms due to airflow limitation. As such, tiotropium was associated with a reduction in asthma exacerbations (Buhl and Hamelmann 2019).

It may be important to target the right patients that would respond to TRPV4 and P2X3 therapies in these allergen challenge studies. While the P2X3-P2X2/3 antagonist AF-219 demonstrated efficacy on refractory chronic cough, the TRPV4 antagonist GSK2798745 did not reduce refractory chronic cough in a study that enrolled 17 patients (Ludbrook et al. 2019). This could indicate that TRPV4 is not important in chronic cough and airway reflexes, but this could also be due to a lack of selection of TRPV4-responsive patients as the study did not

show target engagement. As ATP can be released by numerous processes, P2X3-pathways may be involved in a greater number of patients than TRPV4.

This also raises the question of which of the TRPV4 channel or P2X3-P2X2/3 receptors would be a better target in airway disease involving the TRPV4-P2X3 axis. Targeting TRPV4 could potentially have a broader spectrum of action than P2X3-P2X2/3, by inducing nerve reflexes via P2X3-P2X2/3 receptors, local bronchoconstriction via P2X4 and potentially airway inflammation via P2X1, P2X4, P2X7 (**Figure 7-5**). Side effects should also be considered. Blockade of P2X3-P2X2/3 receptors with AF-219 (gefapixant) administered orally was associated with a loss of taste in patients (Abdulqawi et al. 2015; Smith et al. 2020). Preclinical studies have suggested that P2X2/3 heterotrimers preferentially mediate taste sensation compared to P2X3 homomers, however it is unclear whether it is the case in human (Dicpinigaitis, McGarvey, and Canning 2020; Smith et al. 2020; Garceau and Chauret 2019). Although the TRPV4 antagonist GSK2798745 did not show efficacy on refractory chronic cough, no effect on taste was mentioned in the report (Ludbrook et al. 2019).

Interestingly, animal studies have implied that TRPV4 is involved in mediating sensations in the context of pain but not at baseline (Sipe et al. 2008). In this thesis, TRPV4 did not produce a reflex bronchospasm in naïve animals. If future experiments confirm that TRPV4 is involved in triggering reflexes via P2X3 in the context of airway inflammation but not in the naïve state, TRPV4 blockade might target more disease-specific processes than P2X3 blockade in endotypes that involve the TRPV4-P2X3 axis.

In addition of TRPV4, the blockade of TRPA1 also prevented the LAR in the rat OVA model (Raemdonck et al. 2012). As for inflammatory pathways, there might be different “neuroendotypes” in airway disease (Belvisi et al. 2016). This raises the question of how to identify these neuroendotypes and associated neurophenotypes. ATP could theoretically be used as a biomarker of TRPV4 activation, however with the caveat that it is not specific to TRPV4 and difficult to measure due to its extreme lability (Burnstock 2007).

Overall, investigating airway nerves in asthma may help to develop new therapies with alternative mechanisms than targeting type-2 immune pathways, which could be involved in patients that do not respond to current therapies. While this thesis has focused on atopic asthma associated with eosinophilic inflammation, airway nerves could also be involved in triggering symptoms in patients presenting low grade inflammation, in atopic as well as nonatopic asthma (Belvisi and Birrell 2017).

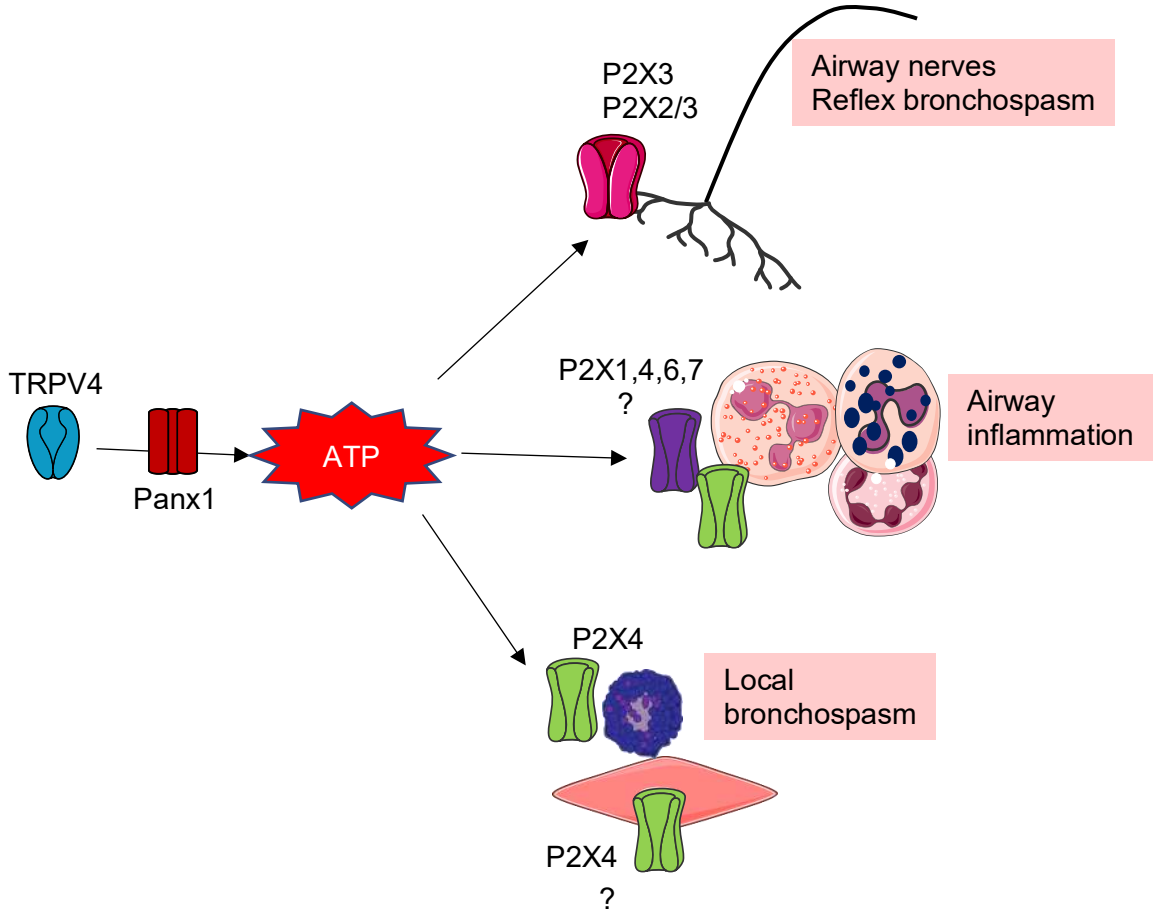


Figure 7-5 Hypothesis of how TRPV4 channels could be involved in asthma

Hypothetical mechanisms by which TRPV4 could be involved in asthma, by inducing the release of ATP which stimulates purinergic receptors and potentially triggers a wide range of effect, with the activation of P2X3-P2X2/3 receptors on airway sensory nerves provoking reflex bronchospasm; the activation of P2X4 receptors provoking local airway smooth muscle contraction; and the activation of P2X1, P2X4 and P2X7 receptors on inflammatory cells provoking airway inflammation.

7.4 Concluding remarks

Data presented in this thesis suggest that TRPV4 activates airway sensory nerves via the release of ATP activating P2X3-P2X2/3 receptors in naïve rats. As TRPV4 and P2X3-P2X2/3 blockade prevented the LAR in a rat OVA model, the TRPV4-P2X3 nerve axis could be a driver of the LAR by activating airway sensory nerves and triggering a cholinergic reflex bronchospasm. However, the TRPV4-P2X3 nerve axis did not seem to elicit reflex bronchospasm in naïve rats. Therefore, further studies are needed to confirm the role of the TRPV4-P2X3 axis in the LAR. Nonetheless, this could suggest that activation of the TRPV4-P2X3 axis provokes a reflex bronchospasm in presence of allergic airway inflammation but not in the naïve state. PAR2 was tested as a potential activator of the TRPV4-P2X3 nerve axis, however two distinct PAR2 antagonists did not inhibit the LAR in the rat OVA model.

The aim of this research was to investigate the role of airway nerves in the LAR, in order to decipher potential mechanisms causing airflow limitation in atopic asthma. The finding that TRPV4 and P2X3-P2X2/3 blockade inhibited the LAR in the rat model supports the hypothesis that airway nerves could play an important role in triggering the LAR, and by extent airflow limitation in atopic asthma. Furthermore, TRPV4 and P2X3-P2X2/3 blockade also reduced allergic airway inflammation, emphasizing that airway nerves could also actively contribute in driving inflammatory processes in asthma. Therefore, this thesis highlights TRPV4 and P2X3-P2X2/3 receptors as potential targets for atopic asthma therapies. Investigating airway nerves could help to develop new asthma therapies targeting alternative pathways than type-2 inflammation to which an important number of patients do not respond.

8 References

Abdulqawi, R., R. Dockry, K. Holt, G. Layton, B. G. Mccarthy, A. P. Ford, and J. A. Smith. 2015. “P2X3 Receptor Antagonist (AF-219) in Refractory Chronic Cough: A Randomised, Double-Blind, Placebo-Controlled Phase 2 Study.” *The Lancet* 385 (9974): 1198–1205. https://doi.org/10.1016/S0140-6736(14)61255-1.

Abey, H. T., D. P. Fairlie, J. D. Moffatt, R. W. Balzary, and T. M. Cocks. 2006. “Protease-Activated Receptor-2 Peptides Activate Neurokinin-1 Receptors in the Mouse Isolated Trachea.” *Journal of Pharmacology and Experimental Therapeutics* 317 (2): 598–605. https://doi.org/10.1124/jpet.105.097121.

Adams, M. N., C. N. Pagel, E. J. MacKie, and J. D. Hooper. 2012. “Evaluation of Antibodies Directed against Human Protease-Activated Receptor-2.” *Naunyn-Schmiedeberg’s Archives of Pharmacology* 385 (9): 861–73. https://doi.org/10.1007/s00210-012-0783-6.

Adams, M. N., R. Ramachandran, M.K. Yau, J. Y. Suen, D. P. Fairlie, M. D. Hollenberg, and J. D. Hooper. 2011. “Structure, Function and Pathophysiology of Protease Activated Receptors.” *Pharmacology and Therapeutics* 130 (3): 248–82. https://doi.org/10.1016/j.pharmthera.2011.01.003.

Adcock, J. J. 1999. “The Relationship between Hyperalgesia and Hyperresponsiveness in the Lung.” *Pain and Neurogenic Inflammation*, 195–206. https://doi.org/10.1007/978-3-0348-8753-3_10.

Adcock, J. J., M. A. Birrell, S. A. Maher, S. J. Bonvini, E. Dubuis, M. A. Wortley, K. E. Baker, and M. G. Belvisi. 2014. “Making Sense Of Sensory Nerves: An In Vivo Characterisation Of Aδ- And C-Fibres Innervating Guinea-Pig Airways.” *Am J Respir Crit Care Med* 189: A3969. https://www.atsjournals.org/doi/abs/10.1164/ajrccm-conference.2014.189.1_MeetingAbstracts.A3969.

Adcock, J. J, G. J. Douglas, M. Garabette, M. Gascoigne, G. Beatch, M. Walker, and C. P. Page. 2003. “RSD931, a Novel Anti-Tussive Agent Acting on Airway Sensory Nerves.” *British Journal of Pharmacology* 138 (3): 407–16. https://doi.org/10.1038/sj.bjp.0705056.

Adner, M., B. J. Canning, H. Meurs, W. Ford, P. Ramos Ramírez, M. P. M. Van Den Berg, M. A. Birrell, et al. 2020. “Back to the Future: Re-Establishing Guinea Pig in Vivo Asthma Models.” *Clinical Science* 134 (11): 1219–42. https://doi.org/10.1042/CS20200394.

Adrian, K., M. K. Bernhard, H. G. Breitinger, and A. Ogilvie. 2000. “Expression of Purinergic Receptors (Ionotropic P2X1-7 and Metabotropic P2Y1-11) during Myeloid Differentiation of HL60 Cells.” *Biochimica et Biophysica Acta-Gene Structure and Expression* 1492 (1): 127–38. https://doi.org/10.1016/S0167-4781(00)00094-4.

Aizawa, N., J.J. Wyndaele, Y. Homma, and Y. Igawa. 2012. “Effects of TRPV4 Cation Channel Activation on the Primary Bladder Afferent Activities of the Rat.” *Neurourology and Urodynamics* 31 (1): 148–55. https://doi.org/10.1002/nau.21212.

Akram, K. M., L. L. Yates, R. Mongey, S. Rothery, D. C. A. Gaboriau, J. Sanderson, M. Hind, M. Griffiths, and C. H. Dean. 2019. “Live Imaging of Alveologenesis in Precision-Cut Lung Slices Reveals Dynamic Epithelial Cell Behaviour.” *Nature Communications* 10 (1): 1–16. https://doi.org/10.1038/s41467-019-09067-3.

Al-Sajee, Dhuha, Roma Sehmi, Thomas J. Hawke, Amani El-Gammal, Karen J. Howie, Richard M. Watson, Marco Londei, Gail M. Gauvreau, and Paul M. O’Byrne. 2018. “Expression of IL-33 and TSLP and Their Receptors in Asthmatic Airways after Inhaled Allergen Challenge.” *American Journal of Respiratory and Critical Care Medicine* 198 (6): 805–7. https://doi.org/10.1164/rccm.201712-2468LE.

Alessandri-Haber, N., O. A. Dina, E. K. Joseph, D. Reichling, and J. D. Levine. 2006. “A Transient Receptor Potential Vanilloid 4-Dependent Mechanism of Hyperalgesia Is Engaged by Concerted Action of Inflammatory Mediators.” *Journal of Neuroscience* 26 (14): 3864–74. https://doi.org/10.1523/JNEUROSCI.5385-05.2006.

Alessandri-Haber, N., J. J. Yeh, A. E. Boyd, C. A. Parada, X. Chen, D. B. Reichling, and J. D. Levine. 2003. “Hypotonicity Induces TRPV4-Mediated Nociception in Rat.” *Neuron* 39 (3): 497–511. https://doi.org/10.1016/S0896-6273(03)00462-8.

Alexander, R., A. Kerby, A. A. Aubdool, A. R. Power, S. Grover, C. Gentry, and A. D. Grant. 2013. “4α-Phorbol 12,13-Didecanoate Activates Cultured Mouse Dorsal Root Ganglia Neurons Independently of TRPV4.” *British Journal of Pharmacology* 168 (3): 761–72. https://doi.org/10.1111/j.1476-5381.2012.02186.x.

Ali, F. R., W. L. G. Oldfield, N. Higashi, M. Larché, and A. B. Kay. 2004. “Late Asthmatic Reactions Induced by Inhalation of Allergen-Derived T Cell Peptides.” *American Journal of Respiratory and Critical Care Medicine* 169 (1): 20–26. https://doi.org/10.1164/rccm.200305-690OC.

Allard, B., I. Bara, G. Gilbert, G. Carvalho, T. Trian, A. Ozier, J. Gillibert-Duplantier, et al. 2014. “Protease Activated Receptor-2 Expression and Function in Asthmatic Bronchial Smooth Muscle.” *PLoS ONE* 9 (2). https://doi.org/10.1371/journal.pone.0086945.

Alvarez, D. F., J. A. King, D. Weber, E. Addison, W. Liedtke, and M. I. Townsley. 2006. “Transient Receptor Potential Vanilloid 4-Mediated Disruption of the Alveolar Septal Barrier: A Novel Mechanism of Acute Lung Injury.” *Circulation Research* 99 (9): 988–95. https://doi.org/10.1161/01.RES.0000247065.11756.19.

Alvarez, M. J., José M. Olaguibel Rivera, B. E. García, A. Rodríguez, A. I. Tabar, and E. Urbiola. 2000. “Airway Inflammation in Asthma and Perennial Allergic Rhinitis. Relationship with Nonspecific Bronchial Responsiveness and Maximal Airway Narrowing.” *Allergy: European Journal of Allergy and Clinical Immunology* 55 (4): 355–62. https://doi.org/10.1034/j.1398-9995.2000.00312.x.

Amadesi, S., J. Nie, N. Vergnolle, G. S. Cottrell, E. F. Grady, M. Trevisani, C. Manni, et al. 2004. “Protease-Activated Receptor 2 Sensitizes the Capsaicin Receptor Transient Receptor Potential Vanilloid Receptor 1 to Induce Hyperalgesia.” *Journal of Neuroscience* 24 (18): 4300–4312. https://doi.org/10.1523/JNEUROSCI.5679-03.2004.

Andrè, E., B. Campi, S. Materazzi, M. Trevisani, S. Amadesi, D. Massi, C. Creminon, et al. 2008. “Cigarette Smoke–Induced Neurogenic Inflammation Is Mediated by α,β-Unsaturated Aldehydes and the TRPA1 Receptor in Rodents.” *Journal of Clinical Investigation*, June. https://doi.org/10.1172/jci34886.

Antonioli, L., C. Blandizzi, P. Pacher, and G. Haskó. 2019. “The Purinergic System as a Pharmacological Target for the Treatment of Immune-Mediated Inflammatory Diseases.” *Pharmacological Reviews* 71 (3): 345–82. https://doi.org/10.1124/pr.117.014878.

Asaduzzaman, M., C. Davidson, D. Nahirney, Y. Fiteih, L. Puttagunta, and H. Vliagoftis. 2018. “Proteinase-Activated Receptor-2 Blockade Inhibits Changes Seen in a Chronic Murine Asthma Model.” *Allergy: European Journal of Allergy and Clinical Immunology* 73 (2): 416–20. https://doi.org/10.1111/all.13313.

Asaduzzaman, M., A. Nadeem, N. Arizmendi, C. Davidson, H. L. Nichols, M. Abel, L. I. Ionescu, et al. 2015. “Functional Inhibition of PAR2 Alleviates Allergen-Induced Airway Hyperresponsiveness and Inflammation.” *Clinical and Experimental Allergy* 45 (12): 1844–55. https://doi.org/10.1111/cea.12628.

Aubier, M., G. Thabut, F. Hamidi, N. Guillou, J. Brard, M-C. Dombret, K. Borensztajn, et al.

2016. “Airway Smooth Muscle Enlargement Is Associated with Protease-Activated Receptor 2/Ligand Overexpression in Patients with Difficult-to-Control Severe Asthma.” *The Journal of Allergy and Clinical Immunology* 138 (3): 729-739.e11. https://doi.org/10.1016/j.jaci.2015.12.1332.

Aun, M.V., R. Bonamichi-Santos, F. M. Arantes-Costa, J. Kalil, and P. Giavina-Bianchi. 2018. “Animal Models of Asthma: Utility and Limitations.” *Journal of Asthma and Allergy* 10 (November): 293–301. https://doi.org/10.2147/JAA.S121092.

Bachelet, C-M., J-F. Bernaudin, and J. Fleury-Feith. 1988. “Distribution and Histochemical Characterization of Pulmonary Mast Cells in the Rat and Guinea Pig.” *International Archives of Allergy and Immunology* 87 (3): 225–29. https://doi.org/10.1159/000234677.

Bagher, M., A. K. Larsson-Callerfelt, O. Rosmark, O. Hallgren, L. Bjermer, and G. Westergren-Thorsson. 2018. “Mast Cells and Mast Cell Tryptase Enhance Migration of Human Lung Fibroblasts through Protease-Activated Receptor 2.” *Cell Communication and Signaling* 16 (1): 59. https://doi.org/10.1186/s12964-018-0269-3.

Baker, K., K. Raemdonck, B. Dekkak, R. J. Snelgrove, J. Ford, F. Shala, M. G. Belvisi, and M. A. Birrell. 2016. “Role of the Ion Channel, Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1), in Allergic Asthma.” *Respiratory Research* 17 (1). https://doi.org/10.1186/s12931-016-0384-x.

Baker, K., K. Raemdonck, R. J. Snelgrove, M. G. Belvisi, and M. A. Birrell. 2017. “Characterisation of a Murine Model of the Late Asthmatic Response.” *Respiratory Research* 18 (1): 55. https://doi.org/10.1186/s12931-017-0541-x.

Barnes, P. J. 2017. “Cellular and Molecular Mechanisms of Asthma and COPD.” *Clinical Science* 131 (13): 1541–58. https://doi.org/10.1042/CS20160487.

Barnes, P. J., J. N. Baraniuk, and M. G. Belvisi. 1991. “Neuropeptides in the Respiratory Tract: Part I.” *American Review of Respiratory Disease*, 1991. https://doi.org/10.1164/ajrccm/144.5.1187.

Barnett, M. W., and P. M. Larkman. 2007. “The Action Potential.” *Practical Neurology* 7 (3): 192–97. https://doi.org/10.1016/b978-0-12-800883-6.00024-0.

Barrios, J., K. R. Patel, L. Aven, R. Achey, M. S. Minns, Y. Lee, V. E. Trinkaus-Randall, and X. Ai. 2017. “Early Life Allergen-Induced Mucus Overproduction Requires Augmented Neural Stimulation of Pulmonary Neuroendocrine Cell Secretion.” *FASEB Journal* 31 (9): 4117–28. https://doi.org/10.1096/fj.201700115R.

Bartlett, D., P. Jeffery, G. Sant’ambrogio, and J. C. Wise. 1976. “Location of Stretch Receptors in the Trachea and Bronchi of the Dog.” *The Journal of Physiology* 258 (2): 409–20. https://doi.org/10.1113/jphysiol.1976.sp011427.

Bartlett, D., G. Sant’ambrogio, and J. C. Wise. 1976. “Transduction Properties of Tracheal Stretch Receptors.” *The Journal of Physiology* 258 (2): 421–32. https://doi.org/10.1113/jphysiol.1976.sp011428.

Basoglu, O. K., A. Pelleg, S. Essilfie-Quaye, C. Brindicci, P. J. Barnes, and S. A. Kharitonov. 2005. “Effects of Aerosolized Adenosine 5′-Triphosphate vs Adenosine 5′-Monophosphate on Dyspnea and Airway Caliber in Healthy Nonsmokers and Patients with Asthma.” *Chest* 128 (4): 1905–9. https://doi.org/10.1378/chest.128.4.1905.

Bates, J. H.T., and C. G. Irvin. 2003. “Measuring Lung Function in Mice: The Phenotyping Uncertainty Principle.” *Journal of Applied Physiology* 94 (4): 1297–1306. https://doi.org/10.1152/japplphysiol.00706.2002.

Bates, J., C. Irvin, V. Brusasco, J. Drazen, J. Fredberg, S. Loring, D. Eidelman, et al. 2004.

"The Use and Misuse of Penh in Animal Models of Lung Disease." *American Journal of Respiratory Cell and Molecular Biology* 31 (3): 373–74. https://doi.org/10.1165/ajrcmb.31.3.1.

Baxter, M., S. Eltom, B. Dekkak, L. Yew-Booth, E. D. Dubuis, S. A. Maher, M. G. Belvisi, and M. A. Birrell. 2014. "Role of Transient Receptor Potential and Pannexin Channels in Cigarette Smoke-Triggered ATP Release in the Lung." *Thorax* 69 (12): 1080–89. https://doi.org/10.1136/thoraxjnl-2014-205467.

Becky Kelly, E., William W. Busse, and Nizar N. Jarjour. 2000. "Inhaled Budesonide Decreases Airway Inflammatory Response to Allergen." *American Journal of Respiratory and Critical Care Medicine* 162 (3): 883–90. https://doi.org/10.1164/ajrccm.162.3.9910077.

Behrsing, H. P., M. J. Furniss, M. Davis, J. E. Tomaszewski, and R. Parchment. 2013. "In Vitro Exposure of Precision-Cut Lung Slices to 2-(4-Amino-3-Methylphenyl)-5-Fluorobenzothiazole Lysylamide Dihydrochloride (NSC 710305, Phortress) Increases Inflammatory Cytokine Content and Tissue Damage." *Toxicological Sciences* 131 (2): 470–79. https://doi.org/10.1093/toxsci/kfs319.

Belvisi, M. G., and M. A. Birrell. 2017. "The Emerging Role of Transient Receptor Potential Channels in Chronic Lung Disease." *European Respiratory Journal* 50 (2). https://doi.org/10.1183/13993003.01357-2016.

Belvisi, M. G., M. A. Birrell, S. Khalid, M. A. Wortley, R. Dockry, J. Coote, K. Holt, et al. 2016. "Neurophenotypes in Airway Diseases: Insights from Translational Cough Studies." *American Journal of Respiratory and Critical Care Medicine* 193 (12): 1364–72. https://doi.org/10.1164/rccm.201508-1602OC.

Belvisi, M. G., M. A. Birrell, M. A. Wortley, S. A. Maher, I. Satia, H. Badri, K. Holt, et al. 2017. "XEN-D0501, a Novel Transient Receptor Potential Vanilloid 1 Antagonist, Does Not Reduce Cough in Patients with Refractory Cough." *American Journal of Respiratory and Critical Care Medicine* 196 (10): 1255–63. https://doi.org/10.1164/rccm.201704-0769OC.

Belvisi, M. G., C. D. Stretton, M. Yacoub, and P. J. Barnes. 1992. "Nitric Oxide Is the Endogenous Neurotransmitter of Bronchodilator Nerves in Humans." *European Journal of Pharmacology* 210 (2): 221–22. https://doi.org/10.1016/0014-2999(92)90676-u.

Bentley, A. M., Q. Meng, D. S. Robinson, Q. Hamid, A. B. Kay, and S. R. Durham. 1993. "Increases in Activated T Lymphocytes, Eosinophils, and Cytokine MRNA Expression for Interleukin-5 and Granulocyte/Macrophage Colony-Stimulating Factor in Bronchial Biopsies after Allergen Inhalation Challenge in Atopic Asthmatics." *American Journal of Respiratory Cell and Molecular Biology* 8 (1): 35–42. https://doi.org/10.1165/ajrcmb/8.1.35.

Bergner, A., and M. J. Sanderson. 2002a. "Acetylcholine-Induced Calcium Signaling and Contraction of Airway Smooth Muscle Cells in Lung Slices." *Journal of General Physiology* 119 (2): 187–98. https://doi.org/10.1085/jgp.119.2.187.

———. 2002b. "ATP Stimulates Ca 2+ Oscillations and Contraction in Airway Smooth Muscle Cells of Mouse Lung Slices." *American Journal of Physiology-Lung Cellular and Molecular Physiology* 283 (6): L1271–79. https://doi.org/10.1152/ajplung.00139.2002.

Bergren, D. R. 1988. "Capsaicin Challenge, Reflex Bronchoconstriction, and Local Action of Substance P." *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 254 (5). https://doi.org/10.1152/ajpregu.1988.254.5.r845.

———. 2001. "Chronic Tobacco Smoke Exposure Increases Airway Sensitivity to Capsaicin in Awake Guinea Pigs." *Journal of Applied Physiology* 90 (2): 695–704.

https://doi.org/10.1152/jappl.2001.90.2.695.

Bergren, D. R., and S. R. Sampson. 1982. “Characterization of Intrapulmonary, Rapidly Adapting Receptors of Guinea Pigs.” *Respiration Physiology* 47 (1): 83–95. https://doi.org/10.1016/0034-5687(82)90094-9.

Bergren, D R. 1997. “Sensory Receptor Activation by Mediators of Defense Reflexes in Guinea-Pig Lungs.” *Respiration Physiology* 108 (3): 195–204. https://doi.org/10.1016/s0034-5687(97)00030-3.

Bertin, S., Y. Aoki-Nonaka, P. R. De Jong, L. L. Nohara, H. Xu, S. R. Stanwood, S. Srikanth, et al. 2014. “The Ion Channel TRPV1 Regulates the Activation and Proinflammatory Properties of CD4+ T Cells.” *Nature Immunology* 15 (11): 1055–63. https://doi.org/10.1038/ni.3009.

Birder, L. A., F. A. Kullmann, H. Lee, S. Barrick, W. De Groat, A. Kanai, and M. Caterina. 2007. “Activation of Urothelial Transient Receptor Potential Vanilloid 4 by 4α-Phorbol 12,13-Didecanoate Contributes to Altered Bladder Reflexes in the Rat.” *Journal of Pharmacology and Experimental Therapeutics* 323 (1): 227–35. https://doi.org/10.1124/jpet.107.125435.

Birrell, M. A., S. J. Bonvini, E. Dubuis, S. A. Maher, M. A. Wortley, M. S. Grace, K. Raemdonck, J. J. Adcock, and M. G. Belvisi. 2014. “Tiotropium Modulates Transient Receptor Potential V1 (TRPV1) in Airway Sensory Nerves: A Beneficial off-Target Effect?” *Journal of Allergy and Clinical Immunology* 133 (3). https://doi.org/10.1016/j.jaci.2013.12.003.

Birrell, M. A., N. Crispino, D. J. Hele, H. J. Patel, M. H. Yacoub, P. J. Barnes, and M. G. Belvisi. 2002. “Effect of Dopamine Receptor Agonists on Sensory Nerve Activity: Possible Therapeutic Targets for the Treatment of Asthma and COPD.” *British Journal of Pharmacology* 136 (4): 620–28. https://doi.org/10.1038/sj.bjp.0704758.

Bjermer, L., C. Lemiere, J. Maspero, S. Weiss, J. Zangrilli, and M. Germinaro. 2016. “Reslizumab for Inadequately Controlled Asthma With Elevated Blood Eosinophil Levels: A Randomized Phase 3 Study.” *Chest* 150 (4): 789–98. https://doi.org/10.1016/j.chest.2016.03.032.

Bleecker, E. R., J. M. FitzGerald, P. Chanez, A. Papi, S. F. Weinstein, P. Barker, S. Sproule, et al. 2016. “Efficacy and Safety of Benralizumab for Patients with Severe Asthma Uncontrolled with High-Dosage Inhaled Corticosteroids and Long-Acting B2-Agonists (SIROCCO): A Randomised, Multicentre, Placebo-Controlled Phase 3 Trial.” *The Lancet* 388 (10056): 2115–27. https://doi.org/10.1016/S0140-6736(16)31324-1.

Bonham, A. C., K. S. Kott, K. Ravi, C. T. Kappagoda, and J. P. Joad. 1996. “Substance P Contributes to Rapidly Adapting Receptor Responses to Pulmonary Venous Congestion in Rabbits.” *Journal of Physiology* 493 (1): 229–38. https://doi.org/10.1113/jphysiol.1996.sp021378.

Bonvini, S. J., M. A. Birrell, E. Dubuis, J. J. Adcock, M. A. Wortley, P. Flajolet, P. Bradding, and M. G. Belvisi. 2020. “Novel Airway Smooth Muscle-Mast Cell Interactions and a Role for the TRPV4-ATP Axis in Non-Atopic Asthma.” *European Respiratory Journal*, April, 1901458. https://doi.org/10.1183/13993003.01458-2019.

Bonvini, S. J., M. A. Birrell, M. S. Grace, S. A. Maher, J. J. Adcock, M. A. Wortley, E. Dubuis, et al. 2016. “Transient Receptor Potential Cation Channel, Subfamily V, Member 4 and Airway Sensory Afferent Activation: Role of Adenosine Triphosphate.” *Journal of Allergy and Clinical Immunology* 138 (1): 249-261.e12. https://doi.org/10.1016/j.jaci.2015.10.044.

Boot, J. D., S. De Haas, S. Tarasevych, C. Roy, L. Wang, D. Amin, J. Cohen, et al. 2007.

"Effect of an NK1/NK2 Receptor Antagonist on Airway Responses and Inflammation to Allergen in Asthma." *American Journal of Respiratory and Critical Care Medicine* 175 (5): 450–57. https://doi.org/10.1164/rccm.200608-1186OC.

Bradding, P., and G. Arthur. 2016. "Mast Cells in Asthma - State of the Art." *Clinical and Experimental Allergy* 46 (2): 194–263. https://doi.org/10.1111/cea.12675.

Bradding, P., Y. Okayama, N. Kambe, and H. Saito. 2003. "Ion Channel Gene Expression in Human Lung, Skin, and Cord Blood-Derived Mast Cells." *Journal of Leukocyte Biology* 73 (5): 614–20. https://doi.org/10.1189/jlb.1202602.

Brierley, S. M., A. J. Page, P. A. Hughes, B. Adam, T. Liebregts, N. J. Cooper, G. Holtmann, W. Liedtke, and L. A. Blackshaw. 2008. "Selective Role for TRPV4 Ion Channels in Visceral Sensory Pathways." *Gastroenterology* 134 (7): 2059–69. https://doi.org/10.1053/j.gastro.2008.01.074.

Brightling, C. E., P. Bradding, F. A. Symon, S. T. Holgate, A. J. Wardlaw, and I. D. Pavord. 2002. "Mast-Cell Infiltration of Airway Smooth Muscle in Asthma." *New England Journal of Medicine* 346 (22): 1699–1705. https://doi.org/10.1056/NEJMoa012705.

Brightling, C. E., F. A. Symon, S. S. Birring, P. Bradding, I. D. Pavord, and A. J. Wardlaw. 2002. "TH2 Cytokine Expression in Bronchoalveolar Lavage Fluid T Lymphocytes and Bronchial Submucosa Is a Feature of Asthma and Eosinophilic Bronchitis." *Journal of Allergy and Clinical Immunology* 110 (6): 899–905. https://doi.org/10.1067/mai.2002.129698.

Brightling, C. E., F. A. Symon, S. S. Birring, P. Bradding, A. J. Wardlaw, and I. D. Pavord. 2003. "Comparison of Airway Immunopathology of Eosinophilic Bronchitis and Asthma." *Thorax* 58 (6): 528–32. https://doi.org/10.1136/thorax.58.6.528.

Brown, S. M., A. Koarai, R. G. Sturton, A. G. Nicholson, P. J. Barnes, and L. E. Donnelly. 2013. "A Role for M2 and M3 Muscarinic Receptors in the Contraction of Rat and Human Small Airways." *European Journal of Pharmacology* 702 (1–3): 109–15. https://doi.org/10.1016/j.ejphar.2013.01.054.

Brusselle, G. G., J. C. Kips, J. H. Tavernier, J. G. Heyden, C. A. Cuvelier, R. A. Pauwels, and H. Bluethmann. 1994. "Attenuation of Allergic Airway Inflammation in IL-4 Deficient Mice." *Clinical & Experimental Allergy* 24 (1): 73–80. https://doi.org/10.1111/j.1365-2222.1994.tb00920.x.

Buckley, J., M. A. Birrell, S. A. Maher, A. T. Nials, D. L. Clarke, and M. G. Belvisi. 2011. "EP4 Receptor as a New Target for Bronchodilator Therapy." *Thorax* 66 (12): 1029–35. https://doi.org/10.1136/thx.2010.158568.

Buhl, R., and E. Hamelmann. 2019. "Future Perspectives of Anticholinergics for the Treatment of Asthma in Adults and Children." *Therapeutics and Clinical Risk Management*. Dove Medical Press Ltd. https://doi.org/10.2147/TCRM.S180890.

Burge, P. S., J. Efthimiou, M. Turner-Warwick, and P. J. Nelmes. 1982. "Double-blind Trials of Inhaled Beclomethasone Diproprionate and Fluocortin Butyl Ester in Allergen-induced Immediate and Late Asthmatic Reactions." *Clinical & Experimental Allergy* 12 (6): 523–31. https://doi.org/10.1111/j.1365-2222.1982.tb02551.x.

Burnstock, Geoffrey. 2007. "Physiology and Pathophysiology of Purinergic Neurotransmission." *Physiological Reviews* 87 (2): 659–797. https://doi.org/10.1152/physrev.00043.2006.

———. 2018. "Purine and Purinergic Receptors." *Brain and Neuroscience Advances* 2: 239821281881749. https://doi.org/10.1177/2398212818817494.

Caceres, A. I., M. Brackmann, M. D. Elia, B. F. Bessac, D. Del Camino, M. D'Amours, J. A. S. Witek, et al. 2009. "A Sensory Neuronal Ion Channel Essential for Airway Inflammation and Hyperreactivity in Asthma." *Proceedings of the National Academy of Sciences of the United States of America* 106 (22): 9099–9104. https://doi.org/10.1073/pnas.0900591106.

Campo, Benjamin A. De, and Peter J. Henry. 2005. "Stimulation of Protease-Activated Receptor-2 Inhibits Airway Eosinophilia, Hyperresponsiveness and Bronchoconstriction in a Murine Model of Allergic Inflammation." *British Journal of Pharmacology* 144 (8): 1100–1108. https://doi.org/10.1038/sj.bjp.0706150.

Canning, B. J. 2006. "Reflex Regulation of Airway Smooth Muscle Tone." *Journal of Applied Physiology* 101 (3): 971–85. https://doi.org/10.1152/japplphysiol.00313.2006.

Canning, B. J., and Y. Chou. 2008. "Using Guinea Pigs in Studies Relevant to Asthma and COPD." *Pulmonary Pharmacology and Therapeutics* 21 (5): 702–20. https://doi.org/10.1016/j.pupt.2008.01.004.

Canning, B. J., and A. Fischer. 2001. "Neural Regulation of Airway Smooth Muscle Tone." *Respiration Physiology* 125 (1–2): 113–27. https://doi.org/10.1016/S0034-5687(00)00208-5.

Canning, B. J., S. B. Mazzone, S. N. Meeker, N. Mori, S. M. Reynolds, and B. J. Undem. 2004. "Identification of the Tracheal and Laryngeal Afferent Neurons Mediating Cough in Anaesthetized Guinea-Pigs." *Journal of Physiology* 557 (2): 543–58. https://doi.org/10.1113/jphysiol.2003.057885.

Canning, B. J., S. M. Reynolds, and S. B. Mazzone. 2001. "Multiple Mechanisms of Reflex Bronchospasm in Guinea Pigs." *Journal of Applied Physiology* 91 (6): 2642–53. https://doi.org/10.1152/jappl.2001.91.6.2642.

Carr, M. J., N. M. Schechter, and B. J. Undem. 2000. "Trypsin-Induced, Neurokinin-Mediated Contraction of Guinea Ping Bronchus." *American Journal of Respiratory and Critical Care Medicine* 162 (5): 1662–67. https://doi.org/10.1164/ajrccm.162.5.9912099.

Carstairs, J. R., A. J. Nimmo, and P. J. Barnes. 1985. "Autoradiographic Visualization of Beta-Adrenoceptor Subtypes in Human Lung." *American Review of Respiratory Disease* 132 (3): 541–47. https://doi.org/10.1164/arrd.1985.132.3.541.

Casarosa, P., T. Bouyssou, S. Germeyer, A. Schnapp, F. Gantner, and M. Pieper. 2009. "Preclinical Evaluation of Long-Acting Muscarinic Antagonists: Comparison of Tiotropium and Investigational Drugs." *Journal of Pharmacology and Experimental Therapeutics* 330 (2): 660–68. https://doi.org/10.1124/jpet.109.152470.

Castro, M., J. Corren, I. D. Pavord, J. Maspero, S. Wenzel, K. F. Rabe, W. W. Busse, et al. 2018. "Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma." *New England Journal of Medicine* 378 (26): 2486–96. https://doi.org/10.1056/NEJMoa1804092.

Castro, M., J. Zangrilli, M. E. Wechsler, E. D. Bateman, G. G. Brusselle, P. Bardin, K. Murphy, J. F. Maspero, C. O'Brien, and S. Korn. 2015. "Reslizumab for Inadequately Controlled Asthma with Elevated Blood Eosinophil Counts: Results from Two Multicentre, Parallel, Double-Blind, Randomised, Placebo-Controlled, Phase 3 Trials." *The Lancet Respiratory Medicine* 3 (5): 355–66. https://doi.org/10.1016/S2213-2600(15)00042-9.

Cazzola, M., S. Centanni, and C. F. Donner. 1998. "Anticholinergic Agents." In *Pulmonary Pharmacology and Therapeutics*, 11:381–92. https://doi.org/10.1006/pupt.1999.0174.

Cenac, N., C. Altier, J. P. Motta, E. D'Aldebert, S. Galeano, G. W. Zamponi, and N. Vergnolle.

2010. “Potentiation of TRPV4 Signalling by Histamine and Serotonin: An Important Mechanism for Visceral Hypersensitivity.” *Gut* 59 (4): 481–88. https://doi.org/10.1136/gut.2009.192567.

Cenac, N., T. Bautzova, P. Le Faouder, N. A. Veldhuis, D. P. Poole, C. Rolland, J. Bertrand, et al. 2015. “Quantification and Potential Functions of Endogenous Agonists of Transient Receptor Potential Channels in Patients with Irritable Bowel Syndrome.” *Gastroenterology* 149 (2): 433-444.e7. https://doi.org/10.1053/j.gastro.2015.04.011.

Chen, Ruchong, Steven G. Smith, Brittany Salter, Amani El-Gammal, John Paul Oliveria, Caitlin Obminski, Rick Watson, Paul M. O’Byrne, Gail M. Gauvreau, and Roma Sehmi. 2017. “Allergen-Induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma.” *American Journal of Respiratory and Critical Care Medicine* 196 (6): 700–712. https://doi.org/10.1164/rccm.201612-2427OC.

Cheung, M., W. Bao, D. J. Behm, C. A. Brooks, M. J. Bury, S. E. Dowdell, H. S. Eidam, et al. 2017. “Discovery of GSK2193874: An Orally Active, Potent, and Selective Blocker of Transient Receptor Potential Vanilloid 4.” *ACS Medicinal Chemistry Letters* 8 (5): 549–54. https://doi.org/10.1021/acsmedchemlett.7b00094.

Chou, Y. L., N. Mori, and B. J. Canning. 2018. “Opposing Effects of Bronchopulmonary C-Fiber Subtypes on Cough in Guinea Pigs.” *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 314 (3): R489–98. https://doi.org/10.1152/ajpregu.00313.2017.

Chou, Y. L., M. D. Scarupa, N. Mori, and B. J. Canning. 2008. “Differential Effects of Airway Afferent Nerve Subtypes on Cough and Respiration in Anesthetized Guinea Pigs.” *American Journal of Physiology - Regulatory Integrative and Comparative Physiology* 295 (5). https://doi.org/10.1152/ajpregu.90382.2008.

Chow, J. M., J. D. Moffatt, and T. M. Cocks. 2000. “Effect of Protease-Activated Receptor (PAR)-1, −2 and −4-Activating Peptides, Thrombin and Trypsin in Rat Isolated Airways.” *British Journal of Pharmacology* 131 (8): 1584–91. https://doi.org/10.1038/sj.bjp.0703738.

Christiansen, S. C., D. Proud, R. B. Sarnoff, U. Juergens, C. G. Cochrane, and B. L. Zuraw. 1992. “Elevation of Tissue Kallikrein and Kinin in the Airways of Asthmatic Subjects after Endobronchial Allergen Challenge.” *The American Review of Respiratory Disease* 145 (4 Pt 1): 900–905. https://doi.org/10.1164/ajrccm/145.4_Pt_1.900.

Chuaychoo, B., M. G. Lee, M. Kollarik, R. Pullmann, and B. J. Undem. 2006. “Evidence for Both Adenosine A1 and A2A Receptors Activating Single Vagal Sensory C-Fibres in Guinea Pig Lungs.” *Journal of Physiology* 575 (2): 481–90. https://doi.org/10.1113/jphysiol.2006.109371.

Chuaychoo, B., M. G. Lee, M. Kollarik, and B. J. Undem. 2005. “Effect of 5-Hydroxytryptamine on Vagal C-Fiber Subtypes in Guinea Pig Lungs.” *Pulmonary Pharmacology and Therapeutics* 18 (4): 269–76. https://doi.org/10.1016/j.pupt.2004.12.010.

Chung, K. F., S. E. Wenzel, J. L. Brozek, A. Bush, M. Castro, P. J. Sterk, I. M. Adcock, et al. 2014. “International ERS/ATS Guidelines on Definition, Evaluation and Treatment of Severe Asthma.” *European Respiratory Journal* 43 (2): 343–73. https://doi.org/10.1183/09031936.00202013.

Cicala, C., D. Spina, S. D. Keir, B. Severino, R. Meli, C. P. Page, and G. Cirino. 2001. “Protective Effect of a PAR2-Activating Peptide on Histamine-Induced Bronchoconstriction in Guinea-Pig.” *British Journal of Pharmacology* 132 (6): 1229–34. https://doi.org/10.1038/sj.bjp.0703934.

Cieslewicz, G., A. Tomkinson, A. Adler, C. Duez, J. Schwarze, K. Takeda, K. A. Larson, J. J. Lee, C. G. Irvin, and E. W. Gelfand. 1999. “The Late, but Not Early, Asthmatic Response Is Dependent on IL-5 and Correlates with Eosinophil Infiltration.” *Journal of Clinical Investigation* 104 (3): 301–8. https://doi.org/10.1172/JCI7010.

Clark, J. M., W. M. Abraham, C. E. Fishman, R. Forteza, A. Ahmed, A. Cortes, R. L. Warne, W. R. Moore, and R. D. Tanaka. 1995. “Tryptase Inhibitors Block Allergen-Induced Airway and Inflammatory Responses in Allergic Sheep.” *American Journal of Respiratory and Critical Care Medicine* 152 (6 I): 2076–83. https://doi.org/10.1164/ajrccm.152.6.8520778.

Cockayne, D. A., P. M. Dunn, Y. Zhong, W. Rong, S. G. Hamilton, G. E. Knight, H. Z. Ruan, et al. 2005. “P2X2 Knockout Mice and P2X2/P2X3 Double Knockout Mice Reveal a Role for the P2X2 Receptor Subunit in Mediating Multiple Sensory Effects of ATP.” *The Journal of Physiology* 567 (Pt 2): 621–39. https://doi.org/10.1113/jphysiol.2005.088435.

Cockayne, D. A., S. G. Hamilton, Q. M. Zhu, P. M. Dunn, Y. Zhong, S. Novakovic, A. B. Malmberg, et al. 2000. “Urinary Bladder Hyporeflexia and Reduced Pain-Related Behaviour in P2X3-Deficient Mice.” *Nature* 407 (6807): 1011–15. https://doi.org/10.1038/35039519.

Cockcroft, D. W. 2014. “Allergen-Induced Asthma.” *Canadian Respiratory Journal* 21 (5): 279–82. https://doi.org/10.1155/2014/719272.

Cockcroft, D. W., and K. Y. Murdock. 1987. “Comparative Effects of Inhaled Salbutamol, Sodium Cromoglycate, and Beclomethasone Dipropionate on Allergen-Induced Early Asthmatic Responses, Late Asthmatic Responses, and Increased Bronchial Responsiveness to Histamine.” *The Journal of Allergy and Clinical Immunology* 79 (5): 734–40. https://doi.org/10.1016/0091-6749(87)90204-1.

Cockcroft, D. W., R. E. Ruffin, and F. E. Hargreave. 1978. “Effect of Sch1000 in Allergen-Induced Asthma.” *Clinical & Experimental Allergy* 8 (4): 361–72. https://doi.org/10.1111/j.1365-2222.1978.tb00471.x.

Cocks, T. M., B. Fong, J. M. Chow, G. P. Anderson, A. G. Frauman, R. G. Goldie, P. J. Henry, M. J. Carr, J. R. Hamilton, and J. D. Moffatt. 1999. “A Protective Role for Protease-Activated Receptors in the Airways.” *Nature* 398 (6723): 156–60. https://doi.org/10.1038/18223.

Cocks, T. M., and J. D. Moffatt. 2001. “Protease-Activated Receptor-2 (PAR2) in the Airways.” *Pulmonary Pharmacology and Therapeutics* 14 (3): 183–91. https://doi.org/10.1006/pupt.2001.0285.

Coddou, C., R. Sandoval, M. J. Hevia, and S. S. Stojilkovic. 2019. “Characterization of the Antagonist Actions of 5-BDBD at the Rat P2X4 Receptor.” *Neuroscience Letters* 690 (January): 219–24. https://doi.org/10.1016/j.neulet.2018.10.047.

Cohn, L., J. S. Tepper, and K. Bottomly. 1998. “IL-4-Independent Induction of Airway Hyperresponsiveness by Th2, but Not Th1, Cells.” *Journal of Immunology (Baltimore, Md. : 1950)* 161 (8): 3813–16. http://www.ncbi.nlm.nih.gov/pubmed/9780144.

Coleridge, H. M., J. C.G. Coleridge, and H. D. Schultz. 1989. “Afferent Pathways Involved in Reflex Regulation of Airway Smooth Muscle.” *Pharmacology and Therapeutics* 42 (1): 1–63. https://doi.org/10.1016/0163-7258(89)90021-1.

Coleridge, J. C., and H. M. Coleridge. 1984. “Afferent Vagal C Fibre Innervation of the Lungs and Airways and Its Functional Significance.” *Reviews of Physiology Biochemistry and Pharmacology* 99: 1–110. https://doi.org/10.1007/bfb0027715.

Collo, G., R. A. North, E. Kawashima, E. Merlo-Pich, S. Neidhart, A. Surprenant, and G. Buell. 1996. “Cloning of P2X5 and P2X6 Receptors and the Distribution and Properties of an Extended Family of ATP-Gated Ion Channels.” *Journal of Neuroscience* 16 (8): 2495–2507. https://doi.org/10.1523/jneurosci.16-08-02495.1996.

Comberiati, P., M. E. Di Cicco, S. D’Elios, and D. G. Peroni. 2017. “How Much Asthma Is Atopic in Children?” *Frontiers in Pediatrics* 5. https://doi.org/10.3389/fped.2017.00122.

Cosmi, Lorenzo, and Francesco Annunziato. 2017. “Group 2 Innate Lymphoid Cells Are the Earliest Recruiters of Eosinophils in Lungs of Patients with Allergic Asthma.” *American Journal of Respiratory and Critical Care Medicine* 196 (6): 666–68. https://doi.org/10.1164/rccm.201704-0799ED.

Costanzo, M. J., S. C. Yabut, H. R. Almond, P. Andrade-Gordon, T. W. Corcoran, L. De Garavilla, J. A. Kauffman, et al. 2003. “Potent, Small-Molecule Inhibitors of Human Mast Cell Tryptase. Antiasthmatic Action of a Dipeptide-Based Transition-State Analogue Containing a Benzothiazole Ketone.” *Journal of Medicinal Chemistry* 46 (18): 3865–76. https://doi.org/10.1021/jm030050p.

Covic, L., A. L. Gresser, J. Talavera, S. Swift, and A. Kuliopulos. 2002. “Activation and Inhibition of G Protein-Coupled Receptors by Cell-Penetrating Membrane-Tethered Peptides.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (2): 643–48. https://doi.org/10.1073/pnas.022460899.

Cyphert, Jaime M., Martina Kovarova, Irving C. Allen, John M. Hartney, Dennis L. Murphy, Jürgen Wess, and Beverly H. Koller. 2009. “Cooperation between Mast Cells and Neurons Is Essential for Antigen-Mediated Bronchoconstriction.” *The Journal of Immunology* 182 (12): 7430–39. https://doi.org/10.4049/jimmunol.0900039.

D’Agostino, G., A. M. Condino, L. Gioglio, F. Zonta, M. Tonini, and A. Barbieri. 2008. “Isolated Porcine Bronchi Provide a Reliable Model for Development of Bronchodilator Anti-Muscarinic Agents for Human Use.” *British Journal of Pharmacology* 154 (8): 1611–18. https://doi.org/10.1038/bjp.2008.208.

Dai, Y., T. Moriyama, T. Higashi, K. Togashi, K. Kobayashi, H. Yamanaka, M. Tominaga, and K. Noguchi. 2004. “Proteinase-Activated Receptor 2-Mediated Potentiation of Transient Receptor Potential Vanilloid Subfamily 1 Activity Reveals a Mechanism for Proteinase-Induced Inflammatory Pain.” *Journal of Neuroscience* 24 (18): 4293–99. https://doi.org/10.1523/JNEUROSCI.0454-04.2004.

Dai, Y., S. Wang, M. Tominaga, S. Yamamoto, T. Fukuoka, T. Higashi, K. Kobayashi, K. Obata, H. Yamanaka, and K. Noguchi. 2007. “Sensitization of TRPA1 by PAR2 Contributes to the Sensation of Inflammatory Pain.” *Journal of Clinical Investigation* 117 (7): 1979–87. https://doi.org/10.1172/JCI30951.

Damera, G., M. Jiang, H. Zhao, H. W. Fogle, W. F. Jester, J. Freire, and R. A. Panettieri. 2010. “Aclidinium Bromide Abrogates Allergen-Induced Hyperresponsiveness and Reduces Eosinophilia in Murine Model of Airway Inflammation.” *European Journal of Pharmacology* 649 (1–3): 349–53. https://doi.org/10.1016/j.ejphar.2010.09.043.

Dandurand, R. J., C. G. Wang, N. C. Phillips, and D. H. Eidelman. 1993. “Responsiveness of Individual Airways to Methacholine in Adult Rat Lung Explants.” *Journal of Applied Physiology* 75 (1): 364–72. https://doi.org/10.1152/jappl.1993.75.1.364.

Darby, W. G., M. S. Grace, S. Baratchi, and P. McIntyre. 2016. “Modulation of TRPV4 by Diverse Mechanisms.” *International Journal of Biochemistry and Cell Biology* 78: 217–28. https://doi.org/10.1016/j.biocel.2016.07.012.

Davidson, C. E., M. Asaduzzaman, N. G. Arizmendi, D. Polley, Y. Wu, J. R. Gordon, M. D.

Hollenberg, L. Cameron, and H. Vliagoftis. 2013. “Proteinase-Activated Receptor-2 Activation Participates in Allergic Sensitization to House Dustmite Allergens in a Murine Model.” *Clinical and Experimental Allergy* 43 (11): 1274–85. https://doi.org/10.1111/cea.12185.

Davis, B. E., C. Illamperuma, G. M. Gauvreau, R. M. Watson, P. M. O’Byrne, F. Deschesnes, L. P. Boulet, and D. W. Cockcroft. 2009. “Single-Dose Desloratadine and Montelukast and Allergen-Induced Late Airway Responses.” *European Respiratory Journal* 33 (6): 1302–8. https://doi.org/10.1183/09031936.00169008.

De Boer, J. D., C. Van’T Veer, I. Stroo, A. J. Van Der Meer, A. F. De Vos, J. S. Van Der Zee, J. J. T. H. Roelofs, and T. Van Der Poll. 2014. “Protease-Activated Receptor-2 Deficient Mice Have Reduced House Dust Mite-Evoked Allergic Lung Inflammation.” *Innate Immunity* 20 (6): 618–25. https://doi.org/10.1177/1753425913503387.

Delescluse, I., H. Mace, and J. J. Adcock. 2012. “Inhibition of Airway Hyperresponsiveness by TRPV1 Antagonists (SB-705498 and PF-04065463) in the Unanaesthetized, Ovalbumin-Sensitized Guinea Pig.” *British Journal of Pharmacology* 166 (6): 1822–32. https://doi.org/10.1111/j.1476-5381.2012.01891.x.

Deng, Z., N. Paknejad, G. Maksaev, M. Sala-Rabanal, C. G. Nichols, R. K. Hite, and P. Yuan. 2018. “Cryo-EM and X-Ray Structures of TRPV4 Reveal Insight into Ion Permeation and Gating Mechanisms.” *Nature Structural and Molecular Biology* 25 (3): 252–60. https://doi.org/10.1038/s41594-018-0037-5.

Dente, F. L., L. Bancalari, E. Bacci, M. L. Bartoli, S. Carnevali, S. Cianchetti, A. Di Franco, et al. 1999. “Effect of a Single Dose of Salmeterol on the Increase in Airway Eosinophils Induced by Allergen Challenge in Asthmatic Subjects.” *Thorax* 54 (7): 622–24. https://doi.org/10.1136/thx.54.7.622.

Devos, F. C., B. Boonen, Y. A. Alpizar, T. Maes, V. Hox, S. Seys, L. Pollaris, et al. 2016. “Neuro-Immune Interactions in Chemical-Induced Airway Hyperreactivity.” *European Respiratory Journal* 48 (2): 380–92. https://doi.org/10.1183/13993003.01778-2015.

Diamant, Z., G. M. Gauvreau, D. W. Cockcroft, L. P. Boulet, P. J. Sterk, F. H. C. de Jongh, B. Dahlén, and P. M. O’Byrne. 2013. “Inhaled Allergen Bronchoprovocation Tests.” *Journal of Allergy and Clinical Immunology* 132 (5): 1045-1055.e6. https://doi.org/10.1016/j.jaci.2013.08.023.

Diamant, Z., D. C. Grootendorst, M. Veselic-Charvat, M. C. Timmers, M. De Smet, J. A. Leff, B. C. Seidenberg, A. H. Zwinderman, I. Peszek, and P. J. Sterk. 1999. “The Effect of Montelukast (MK-0476), a Cysteinyl Leukotriene Receptor Antagonist, on Allergen-Induced Airway Responses and Sputum Cell Counts in Asthma.” *Clinical and Experimental Allergy* 29 (1): 42–51. https://doi.org/10.1046/j.1365-2222.1999.00447.x.

Diamant, Z., P. N. Sidharta, D. Singh, B. J. O’Connor, R. Zuiker, B. R. Leaker, M. Silkey, and J. Dingemanse. 2014. “Setipiprant, a Selective CRTH2 Antagonist, Reduces Allergen-Induced Airway Responses in Allergic Asthmatics.” *Clinical and Experimental Allergy* 44 (8): 1044–52. https://doi.org/10.1111/cea.12357.

Dicpinigaitis, Peter V., Lorcan P. McGarvey, and Brendan J. Canning. 2020. “P2X3-Receptor Antagonists as Potential Antitussives: Summary of Current Clinical Trials in Chronic Cough.” *Lung* 1 (July): 3. https://doi.org/10.1007/s00408-020-00377-8.

Duong, My Linh, Gail Gauvreau, Richard Watson, George Obminski, Tara Strinich, Michelle Evans, Karen Howie, Kieran Killian, and Paul M. O’Byrne. 2007. “The Effects of Inhaled Budesonide and Formoterol in Combination and Alone When given Directly after Allergen Challenge.” *Journal of Allergy and Clinical Immunology* 119 (2): 322–27. https://doi.org/10.1016/j.jaci.2006.10.018.

Durham, S. R., T. H. Lee, O. Cromwell, R. J. Shaw, T. G. Merrett, J. Merrett, P. Cooper, and A. B. Kay. 1984. “Immunologic Studies in Allergen-Induced Late-Phase Asthmatic Reactions.” *The Journal of Allergy and Clinical Immunology* 74 (1): 49–60. https://doi.org/10.1016/0091-6749(84)90086-1.

Fahy, J. V., H. E. Fleming, H. H. Wong, J. T. Liu, J. Q. Su, J. Reimann, R. B. Fick, and H. A. Boushey. 1997. “The Effect of an Anti-IgE Monoclonal Antibody on the Early- and Late-Phase Responses to Allergen Inhalation in Asthmatic Subjects.” *American Journal of Respiratory and Critical Care Medicine* 155 (6): 1828–34. https://doi.org/10.1164/ajrccm.155.6.9196082.

Fantozzi, I., S. Zhang, O. Platoshyn, C. V. Remillard, R. T. Cowling, and J. X. J. Yuan. 2003. “Hypoxia Increases AP-1 Binding Activity by Enhancing Capacitative Ca 2+ Entry in Human Pulmonary Artery Endothelial Cells.” *American Journal of Physiology - Lung Cellular and Molecular Physiology* 285 (6): L1233–45. https://doi.org/10.1152/ajplung.00445.2002.

Fernandes, J., I. M. Lorenzo, Y. N. Andrade, A. Garcia-Elias, S. A. Serra, J. M. Fernández-Fernández, and M. A. Valverde. 2008. “IP3 Sensitizes TRPV4 Channel to the Mechano- and Osmotransducing Messenger 5′-6′-Epoxyeicosatrienoic Acid.” *Journal of Cell Biology* 181 (1): 143–55. https://doi.org/10.1083/jcb.200712058.

FitzGerald, J. M., E. R. Bleecker, P. Nair, S. Korn, K. Ohta, M. Lommatzsch, G. T. Ferguson, et al. 2016. “Benralizumab, an Anti-Interleukin-5 Receptor α Monoclonal Antibody, as Add-on Treatment for Patients with Severe, Uncontrolled, Eosinophilic Asthma (CALIMA): A Randomised, Double-Blind, Placebo-Controlled Phase 3 Trial.” *The Lancet* 388 (10056): 2128–41. https://doi.org/10.1016/S0140-6736(16)31322-8.

Flajolet, P., J. De Alba, K. Raemdonck, M. G. Belvisi, and M. A. Birrell. 2018. “Characterisation of a Chronic Pre-Clinical Model of Allergic Asthma.” *European Respiratory Journal* 52 (suppl 62): PA4413. https://doi.org/10.1183/13993003.congress-2018.pa4413.

Flajolet, P., S. J. Bonvini, B. Dekkak, N. Kobold, J. J. Adcock, P. Bradding, M. G. Belvisi, and M. A. Birrell. 2019. “Investigating Transient Receptor Potential V4 Channel Induced Bronchospasm.” *European Respiratory Journal* 54 (suppl 63): PA3875. https://doi.org/10.1183/13993003.congress-2019.pa3875.

Flores-Soto, E., V. Carbajal, J. Reyes-García, L. M. García-Hernández, A. Figueroa, M. Checa, C. Barajas-López, and L. M. Montaño. 2011. “In Airways ATP Refills Sarcoplasmic Reticulum via P2X Smooth Muscle Receptors and Induces Contraction through P2Y Epithelial Receptors.” *Pflugers Archiv European Journal of Physiology* 461 (2): 261–75. https://doi.org/10.1007/s00424-010-0886-1.

Forsberg, K., and J. A. Karlsson. 1986. “Cough Induced by Stimulation of Capsaicin-Sensitive Sensory Neurons in Conscious Guinea-Pigs.” *Acta Physiologica Scandinavica* 128 (2): 319–20. https://doi.org/10.1111/j.1748-1716.1986.tb07981.x.

Fox, A. J., P. J. Barnes, L. Urban, and A. Dray. 1993. “An in Vitro Study of the Properties of Single Vagal Afferents Innervating Guinea-pig Airways.” *The Journal of Physiology* 469 (1): 21–35. https://doi.org/10.1113/jphysiol.1993.sp019802.

Fryer, A. D., and M. Wills-Karp. 1991. “Dysfunction of M2-Muscarinic Receptors in Pulmonary Parasympathetic Nerves after Antigen Challenge.” *Journal of Applied Physiology* 71 (6): 2255–61. https://doi.org/10.1152/jappl.1991.71.6.2255.

Fuller, R. W., C. M. Dixon, and P. J. Barnes. 1985. “Bronchoconstrictor Response to Inhaled Capsaicin in Humans.” *Journal of Applied Physiology (Bethesda, Md.: 1985)* 58 (4): 1080–84. https://doi.org/10.1152/jappl.1985.58.4.1080.

Fux, M., T. Pecaric-Petkovic, A. Odermatt, O. V. Hausmann, A. Lorentz, S. C. Bischoff, J. C. Virchow, and C. A. Dahinden. 2014. “IL-33 Is a Mediator Rather than a Trigger of the Acute Allergic Response in Humans.” *Allergy: European Journal of Allergy and Clinical Immunology* 69 (2): 216–22. https://doi.org/10.1111/all.12309.

Gallo, V., F. N. Dijk, J. W. Holloway, S. M. Ring, G. H. Koppelman, D. S. Postma, D. P. Strachan, et al. 2017. “TRPA1 Gene Polymorphisms and Childhood Asthma.” *Pediatric Allergy and Immunology* 28 (2): 191–98. https://doi.org/10.1111/pai.12673.

Garceau, Denis, and Nathalie Chauret. 2019. “BLU-5937: A Selective P2X3 Antagonist with Potent Anti-Tussive Effect and No Taste Alteration.” *Pulmonary Pharmacology and Therapeutics* 56: 56–62. https://doi.org/10.1016/j.pupt.2019.03.007.

Gardell, L. R., J. N. Ma, J. G. Seitzberg, A. E. Knapp, H. H. Schiffer, A. Tabatabaei, C. N. Davis, et al. 2008. “Identification and Characterization of Novel Small-Molecule Protease-Activated Receptor 2 Agonists.” *Journal of Pharmacology and Experimental Therapeutics* 327 (3): 799–808. https://doi.org/10.1124/jpet.108.142570.

Gatti, R., E. Andre, S. Amadesi, T. Q. Dinh, A. Fischer, N. W. Bunnett, S. Harrison, P. Geppetti, and M. Trevisani. 2006. “Protease-Activated Receptor-2 Activation Exaggerates TRPV1-Mediated Cough in Guinea Pigs.” *Journal of Applied Physiology* 101 (2): 506–11. https://doi.org/10.1152/japplphysiol.01558.2005.

Gauthier, M., A. Ray, and S. E. Wenzel. 2015. “Evolving Concepts of Asthma.” *American Journal of Respiratory and Critical Care Medicine* 192 (6): 660–68. https://doi.org/10.1164/rccm.201504-0763PP.

Gauvreau, G. M., L. P. Boulet, D. W. Cockcroft, A. Baatjes, J. Cote, F. Deschesnes, B. Davis, et al. 2008. “Antisense Therapy against CCR3 and the Common Beta Chain Attenuates Allergen-Induced Eosinophilic Responses.” *American Journal of Respiratory and Critical Care Medicine* 177 (9): 952–58. https://doi.org/10.1164/rccm.200708-1251OC.

Gauvreau, G. M., L. P. Boulet, D.W. Cockcroft, J. M. Fitzgerald, C. Carlsten, B. E. Davis, F. Deschesnes, et al. 2011. “Effects of Interleukin-13 Blockade on Allergen-Induced Airway Responses in Mild Atopic Asthma.” *American Journal of Respiratory and Critical Care Medicine* 183 (8): 1007–14. https://doi.org/10.1164/rccm.201008-1210OC.

Gauvreau, G. M., J. Doctor, R. M. Watson, M. Jordana, and P. M. O’Byrne. 1996. “Effects of Inhaled Budesonide on Allergen-Induced Airway Responses and Airway Inflammation.” *American Journal of Respiratory and Critical Care Medicine* 154 (5): 1267–71. https://doi.org/10.1164/ajrccm.154.5.8912734.

Gauvreau, G. M., A. I. El-Gammal, and P. M. O’Byrne. 2015. “Allergen-Induced Airway Responses.” *European Respiratory Journal* 46 (3): 819–31. https://doi.org/10.1183/13993003.00536-2015.

Gauvreau, G. M., J. M. FitzGerald, L. P. Boulet, R. M. Watson, L. Hui, H. Villineuve, T. X. Scime, et al. 2018. “The Effects of a CCR3 Inhibitor, AXP1275, on Allergen-Induced Airway Responses in Adults with Mild-to-Moderate Atopic Asthma.” *Clinical and Experimental Allergy* 48 (4): 445–51. https://doi.org/10.1111/cea.13114.

Gauvreau, G. M., J. M. Lee, R. M. Watson, A. M. A. Irani, L. B. Schwartz, and P. M. O’Byrne. 2000. “Increased Numbers of Both Airway Basophils and Mast Cells in Sputum after Allergen Inhalation Challenge of Atopic Asthmatics.” *American Journal of Respiratory and Critical Care Medicine* 161 (5): 1473–78. https://doi.org/10.1164/ajrccm.161.5.9908090.

Gauvreau, G. M., P. M. O’Byrne, L. P. Boulet, Y. Wang, D. Cockcroft, J. Bigler, J. M. FitzGerald, et al. 2014. “Effects of an Anti-TSLP Antibody on Allergen-Induced Asthmatic Responses.” *New England Journal of Medicine* 370 (22): 2102–10.

https://doi.org/10.1056/NEJMoa1402895.

Gauvreau, G. M., R. M. Watson, and P. M. O'Byrne. 1999. "Kinetics of Allergen-Induced Airway Eosinophilic Cytokine Production and Airway Inflammation." *American Journal of Respiratory and Critical Care Medicine* 160 (2): 640–47. https://doi.org/10.1164/ajrccm.160.2.9809130.

Gevaert, T., J. Vriens, A. Segal, W. Everaerts, T. Roskams, K. Talavera, G. Owsianik, et al. 2007. "Deletion of the Transient Receptor Potential Cation Channel TRPV4 Impairs Murine Bladder Voiding." *Journal of Clinical Investigation* 117 (11): 3453–62. https://doi.org/10.1172/JCI31766.

Gever, J. R., R. Soto, R. A. Henningsen, R. S. Martin, D. H. Hackos, S. Panicker, W. Rubas, et al. 2010. "AF-353, a Novel, Potent and Orally Bioavailable P2X3/P2X2/3 Receptor Antagonist." *British Journal of Pharmacology* 160 (6): 1387–98. https://doi.org/10.1111/j.1476-5381.2010.00796.x.

Global Initiative for Asthma. 2019. "Global Strategy for Asthma Management and Prevention." www.ginasthma.org.

Gombedza, F., V. Kondeti, N. Al-Azzam, S. Koppes, E. Duah, P. Patil, M. Hexter, D. Phillips, C. K. Thodeti, and S. Paruchuri. 2017. "Mechanosensitive Transient Receptor Potential Vanilloid 4 Regulates Dermatophagoides Farinae-Induced Airway Remodeling via 2 Distinct Pathways Modulating Matrix Synthesis and Degradation." *FASEB Journal* 31 (4): 1556–70. https://doi.org/10.1096/fj.201601045R.

Gonzalez, M. Cristina, Patricia Diaz, F. R. Galleguillos, Patricia Ancic, O. Cromwell, and A. B. Kay. 1987. "Allergen-Induced Recruitment of Bronchoalveolar Helper (OKT4) and Suppressor (OKT8) T-Cells in Asthma: Relative Increases in OKT8 Cells in Single Early Responders Compared with Those in Late-Phase Responders." *Http://Dx.Doi.Org/10.1164/Ajrccm/136.3.600* 136: 600–604. https://doi.org/10.1164/AJRCCM/136.3.600.

Gori, S., J. Alcain, S. Vanzulli, M. A. Moreno Ayala, M. Candolfi, C. Jancic, J. Geffner, M. Vermeulen, and G. Salamone. 2019. "Acetylcholine-Treated Murine Dendritic Cells Promote Inflammatory Lung Injury." *PLoS ONE* 14 (3). https://doi.org/10.1371/journal.pone.0212911.

Gori, S., M. Vermeulen, F. Remes-Lenicov, C. Jancic, W. Scordo, A. Ceballos, N. Towstyka, et al. 2017. "Acetylcholine Polarizes Dendritic Cells toward a Th2-Promoting Profile." *Allergy* 72 (2): 221–31. https://doi.org/10.1111/all.12926.

Gorman, M. W., E. O. Feigl, and C. W. Buffington. 2007. "Human Plasma ATP Concentration." *Clinical Chemistry* 53 (2): 318–25. https://doi.org/10.1373/clinchem.2006.076364.

Gosens, R., and N. Gross. 2018. "The Mode of Action of Anticholinergics in Asthma." *European Respiratory Journal* 52 (4). https://doi.org/10.1183/13993003.01247-2017.

Grace, M. S., M. A. Birrell, E. Dubuis, S. A. Maher, and M. G. Belvisi. 2012. "Transient Receptor Potential Channels Mediate the Tussive Response to Prostaglandin E2 and Bradykinin." *Thorax* 67 (10): 891–900. https://doi.org/10.1136/thoraxjnl-2011-201443.

Grace, M. S., E. Dubuis, M. A. Birrell, and M. G. Belvisi. 2013. "Pre-Clinical Studies in Cough Research: Role of Transient Receptor Potential (TRP) Channels." *Pulmonary Pharmacology and Therapeutics* 26 (5): 498–507. https://doi.org/10.1016/j.pupt.2013.02.007.

Grace, M. S., T. Lieu, B. Darby, F. C. Abogadie, N. Veldhuis, N. W. Bunnett, and P. Mcintyre. 2014. "The Tyrosine Kinase Inhibitor Bafetinib Inhibits PAR2-Induced Activation of

TRPV4 Channels in Vitro and Pain in Vivo." *British Journal of Pharmacology* 171 (16): 3881–94. https://doi.org/10.1111/bph.12750.

Grant, Andrew D., Graeme S. Cottrell, Silvia Amadesi, Marcello Trevisani, Paola Nicoletti, Serena Materazzi, Christophe Altier, et al. 2007. "Protease-Activated Receptor 2 Sensitizes the Transient Receptor Potential Vanilloid 4 Ion Channel to Cause Mechanical Hyperalgesia in Mice." *Journal of Physiology* 578 (3): 715–33. https://doi.org/10.1113/jphysiol.2006.121111.

Greene, R., J. Fowler, D. MacGlashan, and D. Weinreich. 1988. "IgE-Challenged Human Lung Mast Cells Excite Vagal Sensory Neurons in Vitro." *Journal of Applied Physiology* 64 (5): 2249–53. https://doi.org/10.1152/jappl.1988.64.5.2249.

Grünig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, et al. 1998. "Requirement for IL-13 Independently of IL-4 in Experimental Asthma." *Science* 282 (5397): 2261–63. https://doi.org/10.1126/science.282.5397.2261.

Gu, Q. D., C. R. Moss, K. L. Kettelhut, C. A. Gilbert, and H. Hu. 2016. "Activation of TRPV4 Regulates Respiration through Indirect Activation of Bronchopulmonary Sensory Neurons." *Frontiers in Physiology* 7: 65. https://doi.org/10.3389/fphys.2016.00065.

Gu, Q., and L. Y. Lee. 2009. "Effect of Protease-Activated Receptor 2 Activation on Single TRPV1 Channel Activities in Rat Vagal Pulmonary Sensory Neurons." *Experimental Physiology* 94 (8): 928–36. https://doi.org/10.1113/expphysiol.2009.047712.

———. 2010. "Regulation of Acid Signaling in Rat Pulmonary Sensory Neurons by Protease-Activated Receptor-2." *American Journal of Physiology - Lung Cellular and Molecular Physiology* 298 (3): L454. https://doi.org/10.1152/ajplung.00381.2009.

Gu, Q., and L.Y. Lee. 2006. "Hypersensitivity of Pulmonary Chemosensitive Neurons Induced by Activation of Protease-Activated Receptor-2 in Rats." *Journal of Physiology* 574 (3): 867–76. https://doi.org/10.1113/jphysiol.2006.110312.

Gui, Yu, Zengyong Wang, Xiao Rui Sun, Michael P. Walsh, Jing Jing Li, Jie Gao, and Xi Long Zheng. 2011. "Uridine Adenosine Tetraphosphate Induces Contraction of Airway Smooth Muscle." *American Journal of Physiology - Lung Cellular and Molecular Physiology* 301 (5): L789-94. https://doi.org/10.1152/ajplung.00203.2011.

Güler, A. D., H. Lee, T. Iida, I. Shimizu, M. Tominaga, and M. Caterina. 2002. "Heat-Evoked Activation of the Ion Channel, TRPV4." *Journal of Neuroscience* 22 (15): 6408–14. https://doi.org/10.1523/jneurosci.22-15-06408.2002.

Gustafsson, B., and C. G. A. Persson. 1989. "Effect of Three Weeks' Treatment with Budesonide on in Vitro Contractile and Relaxant Airway Effects in the Rat." *Thorax* 44 (1): 24–27. https://doi.org/10.1136/thx.44.1.24.

Haldar, P., C. E. Brightling, B. Hargadon, S. Gupta, W. Monteiro, A. Sousa, R. P. Marshall, et al. 2009. "Mepolizumab and Exacerbations of Refractory Eosinophilic Asthma." *New England Journal of Medicine* 360 (10): 973–84. https://doi.org/10.1056/NEJMoa0808991.

Halim, Timotheus Y.F., You Yi Hwang, Seth T. Scanlon, Habib Zaghouani, Natalio Garbi, Padraic G. Fallon, and Andrew N.J. Mckenzie. 2016. "Group 2 Innate Lymphoid Cells License Dendritic Cells to Potentiate Memory TH2 Cell Responses." *Nature Immunology* 17 (1): 57–64. https://doi.org/10.1038/ni.3294.

Halim, Timotheus Y.F., Catherine A. Steer, Laura Mathä, Matthew J. Gold, Itziar Martinez-Gonzalez, Kelly M. McNagny, Andrew N.J. McKenzie, and Fumio Takei. 2014. "Group 2 Innate Lymphoid Cells Are Critical for the Initiation of Adaptive T Helper 2 Cell-Mediated Allergic Lung Inflammation." *Immunity* 40 (3): 425–35.

https://doi.org/10.1016/j.immuni.2014.01.011.

Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. “Noninvasive Measurement of Airway Responsiveness in Allergic Mice Using Barometric Plethysmography.” *American Journal of Respiratory and Critical Care Medicine* 156 (3 I): 766–75. https://doi.org/10.1164/ajrccm.156.3.9606031.

Hamilton, A., I. Faiferman, P. Stober, R. M. Watson, and P. M. O’Byrne. 1998. “Pranlukast, a Cysteinyl Leukotriene Receptor Antagonist, Attenuates Allergen-Induced Early- and Late-Phase Bronchoconstriction and Airway Hyperresponsiveness in Asthmatic Subjects.” *Journal of Allergy and Clinical Immunology* 102 (2): 177–83. https://doi.org/10.1016/S0091-6749(98)70083-1.

Hammad, Hamida, and Bart N. Lambrecht. 2015. “Barrier Epithelial Cells and the Control of Type 2 Immunity.” *Immunity* 43 (1): 29–40. https://doi.org/10.1016/j.immuni.2015.07.007.

Haselden, B. M., A. B. Kay, and M. Larché. 1999. “Immunoglobulin E-Independent Major Histocompatibility Complex- Restricted T Cell Peptide Epitope-Induced Late Asthmatic Reactions.” *Journal of Experimental Medicine* 189 (12): 1885–94. https://doi.org/10.1084/jem.189.12.1885.

Hashimoto, A., H. Maeda, and M. Yokoyama. 1996. “Augmentation of Parasympathetic Nerve Function in Patients with Extrinsic Bronchial Asthma.” *The Kobe Journal of Medical Sciences* 42 (6): 347–59. http://www.ncbi.nlm.nih.gov/pubmed/9153972.

Held, H. D., C. Martin, and S. Uhlig. 1999. “Characterization of Airway and Vascular Responses in Murine Lungs.” *British Journal of Pharmacology* 126 (5): 1191–99. https://doi.org/10.1038/sj.bjp.0702394.

Hele, D. J., M. A. Birrell, S. E. Webber, M. L. Foster, and M. G. Belvisi. 2001. “Mediator Involvement in Antigen-Induced Bronchospasm and Microvascular Leakage in the Airways of Ovalbumin Sensitized Brown Norway Rats.” *British Journal of Pharmacology* 132 (2): 481–88. https://doi.org/10.1038/sj.bjp.0703847.

Hennessey, J. C., and J. J. McGuire. 2013. “Attenuated Vasodilator Effectiveness of Protease-Activated Receptor 2 Agonist in Heterozygous Par2 Knockout Mice.” *PLoS ONE* 8 (2): e55965. https://doi.org/10.1371/journal.pone.0055965.

Heuberger, D. M., and R. A. Schuepbach. 2019. “Protease-Activated Receptors (PARs): Mechanisms of Action and Potential Therapeutic Modulators in PAR-Driven Inflammatory Diseases.” *Thrombosis Journal* 17 (1): 4. https://doi.org/10.1186/s12959-019-0194-8.

Hilvering, B., and I. D. Pavord. 2016. “Biologicals to Treat Allergy and Asthma.” *Encyclopedia of Immunobiology* 5: 321–27. https://doi.org/10.1016/B978-0-12-374279-7.16014-X.

Hirn, S., N. Haberl, K. Loza, M. Epple, W. G. Kreyling, B. Rothen-Rutishauser, M. Rehberg, and F. Krombach. 2014. “Proinflammatory and Cytotoxic Response to Nanoparticles in Precision-Cut Lung Slices.” *Beilstein Journal of Nanotechnology* 5 (1): 2440–49. https://doi.org/10.3762/bjnano.5.253.

Ho, C. Y., Q. Gu, Y. S. Lin, and L. Y. Lee. 2001. “Sensitivity of Vagal Afferent Endings to Chemical Irritants in the Rat Lung.” *Respiration Physiology* 127 (2–3): 113–24. https://doi.org/10.1016/s0034-5687(01)00241-9.

Holgate, S., T. Casale, S. Wenzel, J. Bousquet, Y. Deniz, and C. Reisner. 2005. “The Anti-Inflammatory Effects of Omalizumab Confirm the Central Role of IgE in Allergic Inflammation.” *Journal of Allergy and Clinical Immunology* 115 (3): 459–65. https://doi.org/10.1016/j.jaci.2004.11.053.

Hollenberg, M. D., K. Mihara, D. Polley, J. Y. Suen, A. Han, D. P. Fairlie, and R.

Ramachandran. 2014. “Biased Signalling and Proteinase-Activated Receptors (PARs): Targeting Inflammatory Disease.” *British Journal of Pharmacology* 171 (5): 1180–94. https://doi.org/10.1111/bph.12544.

Hollenberg, M. D., B. Renaux, E. Hyun, S. Houle, N. Vergnolle, M. Saifeddine, and R. Ramachandran. 2008. “Derivatized 2-Furoyl-LIGRLO-Amide, a Versatile and Selective Probe for Proteinase-Activated Receptor 2: Binding and Visualization.” *Journal of Pharmacology and Experimental Therapeutics* 326 (2): 453–62. https://doi.org/10.1124/jpet.108.136432.

Hollenberg, M. D., M. Saifeddine, B. Al-Ani, and A. Kawabata. 1997. “Proteinase-Activated Receptors: Structural Requirements for Activity, Receptor Cross-Reactivity, and Receptor Selectivity of Receptor-Activating Peptides.” *Canadian Journal of Physiology and Pharmacology* 75 (7): 832–41. http://www.ncbi.nlm.nih.gov/pubmed/9315351.

Hox, V., J. A. Vanoirbeek, Y. A. Alpizar, S. Voedisch, I. Callebaut, S. Bobic, A. Sharify, et al. 2013. “Crucial Role of Transient Receptor Potential Ankyrin 1 and Mast Cells in Induction of Nonallergic Airway Hyperreactivity in Mice.” *American Journal of Respiratory and Critical Care Medicine* 187 (5): 486–93. https://doi.org/10.1164/rccm.201208-1358OC.

Hunter, D. D., and B. J. Undem. 1999. “Identification and Substance P Content of Vagal Afferent Neurons Innervating the Epithelium of the Guinea Pig Trachea.” *American Journal of Respiratory and Critical Care Medicine* 159 (6): 1943–48. https://doi.org/10.1164/ajrccm.159.6.9808078.

Idzko, M., H. Hammad, M. van Nimwegen, M. Kool, M. A. M. Willart, F. Muskens, H. C. Hoogsteden, et al. 2007. “Extracellular ATP Triggers and Maintains Asthmatic Airway Inflammation by Activating Dendritic Cells.” *Nature Medicine* 13 (8): 913–19. https://doi.org/10.1038/nm1617.

Inman, M. D., R. M. Watson, T. Rerecich, G. M. Gauvreau, B. N. Lutsky, P. Stryszak, and P. M. O’Byrne. 2001. “Dose-Dependent Effects of Inhaled Mometasone Furoate on Airway Function and Inflammation after Allergen Inhalation Challenge.” *American Journal of Respiratory and Critical Care Medicine* 164 (4): 569–74. https://doi.org/10.1164/ajrccm.164.4.2007063.

Ishimura, M., M. Suda, K. Morizumi, S. Kataoka, T. Maeda, S. Kurokawa, and Y. Hiyama. 2008. “Effects of KP-496, a Novel Dual Antagonist at the Cysteinyl Leukotriene Receptor 1 and the Thromboxane A 2 Receptor, on Airway Obstruction in Guinea Pigs.” *British Journal of Pharmacology* 153 (4): 669–75. https://doi.org/10.1038/sj.bjp.0707602.

Isogai, S., A. Athiviraham, R. S. Fraser, R. Taha, Q. Hamid, and J. G. Martin. 2007. “Interferon-γ-Dependent Inhibition of Late Allergic Airway Responses and Eosinophilia by CD8+γδ T Cells.” *Immunology* 122 (2): 230–38. https://doi.org/10.1111/j.1365-2567.2007.02632.x.

Isogai, S., S. Jedrzkiewicz, R. Taha, Q. Hamid, and J. G. Martin. 2005. “Resident CD8+ T Cells Suppress CD4+ T Cell-Dependent Late Allergic Airway Responses.” *Journal of Allergy and Clinical Immunology* 115 (3): 521–26. https://doi.org/10.1016/j.jaci.2004.11.036.

Isogai, S., A. Rubin, K. Maghni, D. Ramos-Barbon, R. Taha, Y. Yoshizawa, Q. Hamid, and J. G. Martin. 2003. “The Effects of CD8+gammadelta T Cells on Late Allergic Airway Responses and Airway Inflammation in Rats.” *The Journal of Allergy and Clinical Immunology* 112 (3): 547–55. https://doi.org/10.1016/s0091-6749(03)01720-2.

Isogai, S., R. Taha, M. Tamaoka, Y. Yoshizawa, Q. Hamid, and J. G. Martin. 2004. “CD8 + Aβ T Cells Can Mediate Late Airway Responses and Airway Eosinophilia in Rats.” *Journal of Allergy and Clinical Immunology* 114 (6): 1345–52.

https://doi.org/10.1016/j.jaci.2004.09.021.

James, S. L., D. Abate, K. H. Abate, S. M. Abay, C. Abbafati, N. Abbasi, H. Abbastabar, et al. 2018. “Global, Regional, and National Incidence, Prevalence, and Years Lived with Disability for 354 Diseases and Injuries for 195 Countries and Territories, 1990-2017: A Systematic Analysis for the Global Burden of Disease Study 2017.” *The Lancet* 392 (10159): 1789–1858. https://doi.org/10.1016/S0140-6736(18)32279-7.

Jammes, Y., and N. Mei. 1979. “Assessment of the Pulmonary Origin of Bronchoconstrictor Vagal Tone.” *The Journal of Physiology* 291 (1): 305–16. https://doi.org/10.1113/jphysiol.1979.sp012814.

Jarjour, N N, W J Calhoun, L B Schwartz, and W W Busse. 1991. “Elevated Bronchoalveolar Lavage Fluid Histamine Levels in Allergic Asthmatics Are Associated with Increased Airway Obstruction.” *The American Review of Respiratory Disease* 144 (1): 83–87. https://doi.org/10.1164/ajrccm/144.1.83.

Jeffery, P., and L. Reid. 1973. “Intra-Epithelial Nerves in Normal Rat Airways: A Quantitative Electron Microscopic Study.” *Journal of Anatomy* 114 (Pt 1): 35–45. http://www.ncbi.nlm.nih.gov/pubmed/4714939.

Jia, Y., X. Wang, L. A. Varty, C. A. Rizzo, R. Yang, C. C. Correll, P. T. Phelps, R. W. Egan, and J. A. Hey. 2004. “Functional TRPV4 Channels Are Expressed in Human Airway Smooth Muscle Cells.” *American Journal of Physiology - Lung Cellular and Molecular Physiology* 287 (2): L272-8. https://doi.org/10.1152/ajplung.00393.2003.

John-Schuster, G., S. de Kleijn, Y. van Wijck, V. Kremer, H. H. Smits, M. P. Pieper, P. S. Hiemstra, and C. Taube. 2017. “The Effect of Tiotropium in Combination with Olodaterol on House Dust Mite-Induced Allergic Airway Disease.” *Pulmonary Pharmacology and Therapeutics* 45: 210–17. https://doi.org/10.1016/j.pupt.2017.06.010.

Johnson, J. R., R. E. Wiley, R. Fattouh, F. K. Swirski, B. U. Gajewska, A. J. Coyle, J. C. Gutierrez-Ramos, R. Ellis, M. D. Inman, and M. Jordana. 2004. “Continuous Exposure to House Dust Mite Elicits Chronic Airway Inflammation and Structural Remodeling.” *American Journal of Respiratory and Critical Care Medicine* 169 (3): 378–85. https://doi.org/10.1164/rccm.200308-1094oc.

Junger, W. G. 2011. “Immune Cell Regulation by Autocrine Purinergic Signalling.” *Nature Reviews Immunology* 11 (3): 201–12. https://doi.org/10.1038/nri2938.

Kajekar, R., D. Proud, A. C. Myers, S. N. Meeker, and B. J. Undem. 1999. “Characterization of Vagal Afferent Subtypes Stimulated by Bradykinin in Guinea Pig Trachea.” *The Journal of Pharmacology and Experimental Therapeutics* 289 (2): 682–87. http://www.ncbi.nlm.nih.gov/pubmed/10215640.

Kappagoda, C. T., and K. Ravi. 1989. “Plasmapheresis Affects Responses of Slowly and Rapidly Adapting Airway Receptors to Pulmonary Venous Congestion in Dogs.” *The Journal of Physiology* 416 (1): 79–91. https://doi.org/10.1113/jphysiol.1989.sp017750.

Kawabata, Atsufumi, Toru Kanke, Daiki Yonezawa, Tsuyoshi Ishiki, Masako Saka, Mototsugu Kabeya, Fumiko Sekiguchi, et al. 2004. “Potent and Metabolically Stable Agonists for Protease-Activated Receptor-2: Evaluation of Activity in Multiple Assay Systems in Vitro and in Vivo.” *Journal of Pharmacology and Experimental Therapeutics* 309 (3): 1098–1107. https://doi.org/10.1124/jpet.103.061010.

Kay, A. B., and A. Menzies-Gow. 2003. “Eosinophils and Interleukin-5.” *American Journal of Respiratory and Critical Care Medicine* 167 (12): 1586–87. https://doi.org/10.1164/rccm.2304001.

Kerstjens, H. A. M., T. B. Casale, E. R. Bleecker, E. O. Meltzer, E. Pizzichini, O. Schmidt, M. Engel, et al. 2015. “Tiotropium or Salmeterol as Add-on Therapy to Inhaled Corticosteroids for Patients with Moderate Symptomatic Asthma: Two Replicate, Double-Blind, Placebo-Controlled, Parallel-Group, Active-Comparator, Randomised Trials.” *The Lancet Respiratory Medicine* 3 (5): 367–76. https://doi.org/10.1016/S2213-2600(15)00031-4.

Kerstjens, H. A. M., M. Engel, R. Dahl, P. Paggiaro, E. Beck, M. Vandewalker, R. Sigmund, W. Seibold, P. Moroni-Zentgraf, and E. D. Bateman. 2012. “Tiotropium in Asthma Poorly Controlled with Standard Combination Therapy.” *New England Journal of Medicine* 367 (13): 1198–1207. https://doi.org/10.1056/NEJMoa1208606.

Kesler, B. S., and B. J. Canning. 1999. “Regulation of Baseline Cholinergic Tone in Guinea-Pig Airway Smooth Muscle.” *The Journal of Physiology* 518 (Pt 3): 843–55. https://doi.org/10.1111/j.1469-7793.1999.0843p.x.

Khan, L. N., O. M. Kon, A. J. Macfarlane, Q. Meng, S. Ying, N. C. Barnes, and A. B. Kay. 2000. “Attenuation of the Allergen-Induced Late Asthmatic Reaction by Cyclosporin A Is Associated with Inhibition of Bronchial Eosinophils, Interleukin-5, Granulocyte Macrophage Colony-Stimulating Factor, and Eotaxin.” *American Journal of Respiratory and Critical Care Medicine* 162 (4): 1377–82. https://doi.org/10.1164/ajrccm.162.4.9911117.

Kidney, J. C., L. P. Boulet, F. E. Hargreave, F. Deschesnes, V. A. Swystun, P. M. O’Byrne, N. Choudry, et al. 1997. “Evaluation of Single-Dose Inhaled Corticosteroid Activity with an Allergen Challenge Model.” *Journal of Allergy and Clinical Immunology* 100 (1): 65–70. https://doi.org/10.1016/S0091-6749(97)70196-9.

Kinoshita, Takashi, Adrian Baatjes, Steven G. Smith, Benny Dua, Richard Watson, Tomotaka Kawayama, Mark Larche, Gail M. Gauvreau, and Paul M. O’Byrne. 2014. “Natural Regulatory T Cells in Isolated Early Responders Compared with Dual Responders with Allergic Asthma.” *Journal of Allergy and Clinical Immunology* 133 (3): 696–703. https://doi.org/10.1016/j.jaci.2013.08.025.

Knight, D. A., S. Lim, A. K. Scaffidi, N. Roche, K. F. Chung, G. A. Stewart, and P. J. Thompson. 2001. “Protease-Activated Receptors in Human Airways: Upregulation of PAR-2 in Respiratory Epithelium from Patients with Asthma.” *Journal of Allergy and Clinical Immunology* 108 (5): 797–803. https://doi.org/10.1067/mai.2001.119025.

Köhler, R., W. T. Heyken, P. Heinau, R. Schubert, H. Si, M. Kacik, C. Busch, I. Grgic, T. Maier, and J. Hoyer. 2006. “Evidence for a Functional Role of Endothelial Transient Receptor Potential V4 in Shear Stress-Induced Vasodilatation.” *Arteriosclerosis, Thrombosis, and Vascular Biology* 26 (7): 1495–1502. https://doi.org/10.1161/01.ATV.0000225698.36212.6a.

Kollarik, M., Q. T. Dinh, A. Fischer, and B. J. Undem. 2003. “Capsaicin-Sensitive and -Insensitive Vagal Bronchopulmonary C-Fibres in the Mouse.” *Journal of Physiology* 551 (3): 869–79. https://doi.org/10.1113/jphysiol.2003.042028.

Kollarik, M., F. Ru, and B. J. Undem. 2019. “Phenotypic Distinctions between the Nodose and Jugular TRPV1-Positive Vagal Sensory Neurons in the Cynomolgus Monkey.” *NeuroReport* 30 (8): 533–37. https://doi.org/10.1097/WNR.0000000000001231.

Kollarik, M., and B. J. Undem. 2002. “Mechanisms of Acid-Induced Activation of Airway Afferent Nerve Fibres in Guinea-Pig.” *Journal of Physiology* 543 (2): 591–600. https://doi.org/10.1113/jphysiol.2002.022848.

Krishna, M. T., A. Chauhan, L. Little, K. Sampson, R. Hawksworth, T. Mant, R. Djukanovic, T. Lee, and S. Holgate. 2001. “Inhibition of Mast Cell Tryptase by Inhaled APC 366

Attenuates Allergen-Induced Late-Phase Airway Obstruction in Asthma." *Journal of Allergy and Clinical Immunology* 107 (6): 1039–45. https://doi.org/10.1067/mai.2001.115631.

Krug, N., J. M. Hohlfeld, A. M. Kirsten, O. Kornmann, K. M. Beeh, D. Kappeler, S. Korn, et al. 2015. "Allergen-Induced Asthmatic Responses Modified by a GATA3-Specific DNAzyme." *New England Journal of Medicine* 372 (21): 1987–95. https://doi.org/10.1056/NEJMoa1411776.

Kumar, R. K., C. Herbert, M. Yang, A. M. L. Koskinen, A. N. J. McKenzie, and P. S. Foster. 2002. "Role of Interleukin-13 in Eosinophil Accumulation and Airway Remodelling in a Mouse Model of Chronic Asthma." *Clinical and Experimental Allergy* 32 (7): 1104–11. https://doi.org/10.1046/j.1365-2222.2002.01420.x.

Kummer, W., and G. Krasteva-Christ. 2014. "Non-Neuronal Cholinergic Airway Epithelium Biology." *Current Opinion in Pharmacology* 16 (1): 43–49. https://doi.org/10.1016/j.coph.2014.03.001.

Kushnir-Sukhov, N. M., J. M. Brown, Y. Wu, A. Kirshenbaum, and D. D. Metcalfe. 2007. "Human Mast Cells Are Capable of Serotonin Synthesis and Release." *Journal of Allergy and Clinical Immunology* 119 (2): 498–99. https://doi.org/10.1016/j.jaci.2006.09.003.

Kwong, K., M. Kollarik, C. Nassenstein, F. Ru, and B. J. Undem. 2008. "P2X2 Receptors Differentiate Placodal vs. Neural Crest C-Fiber Phenotypes Innervating Guinea Pig Lungs and Esophagus." *American Journal of Physiology - Lung Cellular and Molecular Physiology* 295 (5). https://doi.org/10.1152/ajplung.90360.2008.

Kwong, K., and L. Y. Lee. 2002. "PGE2 Sensitizes Cultured Pulmonary Vagal Sensory Neurons to Chemical and Electrical Stimuli." *Journal of Applied Physiology* 93 (4): 1419–28. https://doi.org/10.1152/japplphysiol.00382.2002.

———. 2005. "Prostaglandin E2 Potentiates a TTX-Resistant Sodium Current in Rat Capsaicin-Sensitive Vagal Pulmonary Sensory Neurones." *Journal of Physiology* 564 (2): 437–50. https://doi.org/10.1113/jphysiol.2004.078725.

Kwong, K., C. Nassenstein, L. de Garavilla, S. Meeker, and B. J. Undem. 2010. "Thrombin and Trypsin Directly Activate Vagal C-Fibres in Mouse Lung via Protease-Activated Receptor-1." *Journal of Physiology* 588 (7): 1171–77. https://doi.org/10.1113/jphysiol.2009.181669.

Laitinen, A. 1985. "Ultrastructural Organisation of Intraepithelial Nerves in the Human Airway Tract." *Thorax* 40 (7): 488–92. https://doi.org/10.1136/thx.40.7.488.

Laitinen, L. A., M. Heino, A. Laitinen, T. Kava, and T. Haahtela. 1985. "Damage of the Airway Epithelium and Bronchial Reactivity in Patients with Asthma." *American Review of Respiratory Disease* 131 (4): 599–606. https://doi.org/10.1164/arrd.1985.131.4.599.

Lamb, J. P., and M. P. Sparrow. 2002. "Three-Dimensional Mapping of Sensory Innervation with Substance P in Porcine Bronchial Mucosa." *American Journal of Respiratory and Critical Care Medicine* 166 (9): 1269–81. https://doi.org/10.1164/rccm.2112018.

Lambrecht, B. N., and H. Hammad. 2015. "The Immunology of Asthma." *Nature Immunology* 16 (1): 45–56. https://doi.org/10.1038/ni.3049.

———. 2017. "The Immunology of the Allergy Epidemic and the Hygiene Hypothesis." *Nature Immunology* 18 (10): 1076–83. https://doi.org/10.1038/ni.3829.

Lambrecht, B. N., H. Hammad, and J. V. Fahy. 2019. "The Cytokines of Asthma." *Immunity* 50 (4): 975–91. https://doi.org/10.1016/j.immuni.2019.03.018.

Lambrecht, B. N., M. Kool, M. A. M. Willart, and H. Hammad. 2009. “Mechanism of Action of Clinically Approved Adjuvants.” *Current Opinion in Immunology* 21 (1): 23–29. https://doi.org/10.1016/j.coi.2009.01.004.

Lambrecht, B. N., E. K. Persson, and H. Hammad. 2017. “Myeloid Cells in Asthma.” *Microbiology Spectrum* 5 (1). https://doi.org/10.1128/microbiolspec.MCHD-0053-2016.

Larson, S. D., E. S. Schelegle, D. M. Hyde, and C. G. Plopper. 2003. “The Three-Dimensional Distribution of Nerves along the Entire Intrapulmonary Airway Tree of the Adult Rat and the Anatomical Relationship between Nerves and Neuroepithelial Bodies.” *American Journal of Respiratory Cell and Molecular Biology* 28 (5): 592–99. https://doi.org/10.1165/rcmb.4889.

Laude, E. A., K. S. Higgins, and A. H. Morice. 1993. “A Comparative Study of the Effects of Citric Acid, Capsaicin and Resiniferatoxin on the Cough Challenge in Guinea-Pig and Man.” *Pulmonary Pharmacology* 6 (3): 171–75. https://doi.org/10.1006/pulp.1993.1023.

Leckie, M. J., A. Ten Brinke, J. Khan, Z. Diamant, B. J. O’xonnor, C. M. Walls, A. K. Mathur, et al. 2000. “Effects of an Interleukin-5 Blocking Monoclonal Antibody on Eosinophils, Airway Hyper-Responsiveness, and the Late Asthmatic Response.” *Lancet* 356 (9248): 2144–48. https://doi.org/10.1016/S0140-6736(00)03496-6.

Lee, L. Y., and R. F. Morton. 1993. “Histamine Enhances Vagal Pulmonary C-Fiber Responses to Capsaicin and Lung Inflation.” *Respiration Physiology* 93 (1): 83–96. https://doi.org/10.1016/0034-5687(93)90070-Q.

Lee, L. Y., and T. E. Pisarri. 2001. “Afferent Properties and Reflex Functions of Bronchopulmonary C-Fibers.” *Respiration Physiology* 125 (1–2): 47–65. https://doi.org/10.1016/s0034-5687(00)00204-8.

Lee, M. S., and E. A. Lerner. 2019. “Targeting PAR2 with Pepducins.” *Journal of Investigative Dermatology* 139 (2): 282–84. https://doi.org/10.1016/j.jid.2018.09.008.

Leigh, R., D. Vethanayagam, M. Yoshida, R. M. Watson, T. Rerecich, M. D. Inman, and P. M. O’Byrne. 2002. “Effects of Montelukast and Budesonide on Airway Responses and Airway Inflammation in Asthma.” *American Journal of Respiratory and Critical Care Medicine* 166 (9): 1212–17. https://doi.org/10.1164/rccm.200206-509OC.

Lewis, C., S. Neidhart, C. Holy, R. A. North, G. Buell, and A. Surprenant. 1995. “Coexpression of P2X2 and P2X3 Receptor Subunits Can Account for ATP-Gated Currents in Sensory Neurons.” *Nature* 377 (6548): 432–35. https://doi.org/10.1038/377432a0.

Li, Bobby W.S., Marjolein J.W. de Bruijn, Irma Tindemans, Melanie Lukkes, Alex KleinJan, Henk C. Hoogsteden, and Rudi W. Hendriks. 2016. “T Cells Are Necessary for ILC2 Activation in House Dust Mite-Induced Allergic Airway Inflammation in Mice.” *European Journal of Immunology* 46 (6): 1392–1403. https://doi.org/10.1002/eji.201546119.

Li, J., Y. Chen, Q. Y. Chen, D. Liu, L. Xu, G. Cheng, X. Yang, Z. Guo, and Y. Zeng. 2019. “Role of Transient Receptor Potential Cation Channel Subfamily V Member 1 (TRPV1) on Ozone-Exacerbated Allergic Asthma in Mice.” *Environmental Pollution* 247: 586–94. https://doi.org/10.1016/j.envpol.2019.01.091.

Liebermann-Meffert, D. M., B. Walbrun, C. A. Hiebert, and J. R. Siewert. 1999. “Recurrent and Superior Laryngeal Nerves: A New Look with Implications for the Esophageal Surgeon.” *Annals of Thoracic Surgery* 67 (1): 217–23. https://doi.org/10.1016/S0003-4975(98)01131-X.

Liedtke, W., Y. Choe, M. A. Martí-Renom, A. M. Bell, C. S. Denis, AndrejŠali, A. J. Hudspeth, J. M. Friedman, and S. Heller. 2000. “Vanilloid Receptor-Related Osmotically Activated

Channel (VR-OAC), a Candidate Vertebrate Osmoreceptor." *Cell* 103 (3): 525–35. https://doi.org/10.1016/S0092-8674(00)00143-4.

Liedtke, W., and J. M. Friedman. 2003. "Abnormal Osmotic Regulation in Trpv4-/- Mice." *Proceedings of the National Academy of Sciences of the United States of America* 100 (23): 13698–703. https://doi.org/10.1073/pnas.1735416100.

Lin, C., J. Von Der Thüsen, J. Daalhuisen, M. ten Brink, B. Crestani, T. Van Der Poll, K. Borensztajn, and C. A. Spek. 2015. "Protease-Activated Receptor (PAR)-2 Is Required for PAR-1 Signalling in Pulmonary Fibrosis." *Journal of Cellular and Molecular Medicine* 19 (6): 1346–56. https://doi.org/10.1111/jcmm.12520.

Liu, M. C., W. C. Hubbard, D. Proud, B. A. Stealey, S. J. Galli, A. Kagey-Sobotka, E. R. Bleecker, and L. M. Lichtenstein. 1991. "Immediate and Late Inflammatory Responses to Ragweed Antigen Challenge of the Peripheral Airways in Allergic Asthmatics: Cellular, Mediator, and Permeability Changes." *American Review of Respiratory Disease* 144 (1): 51–58. https://doi.org/10.1164/ajrccm/144.1.51.

Liu, M. C., D. Proud, L. M. Lichtenstein, W. C. Hubbard, B. S. Bochner, B. A. Stealey, L. Breslin, et al. 2001. "Effects of Prednisone on the Cellular Responses and Release of Cytokines and Mediators after Segmental Allergen Challenge of Asthmatic Subjects." *Journal of Allergy and Clinical Immunology* 108 (1): 29–38. https://doi.org/10.1067/mai.2001.116004.

Liu, R., J. Song, H. Li, Z. Wu, H. Chen, W. Wu, and L. Gu. 2014. "Treatment of Canine Asthma by High Selective Vagotomy." *Journal of Thoracic and Cardiovascular Surgery* 148 (2): 683–89. https://doi.org/10.1016/j.jtcvs.2013.12.041.

Liu, S., K. Chihara, and K. Maeyama. 2005. "The Contribution of Mast Cells to the Late-Phase of Allergic Asthma in Rats." *Inflammation Research* 54 (5): 221–28. https://doi.org/10.1007/s00011-005-1346-9.

Lloyd, Clare M., and Robert J. Snelgrove. 2018. "Type 2 Immunity: Expanding Our View." *Science Immunology* 3 (25): 1604. https://doi.org/10.1126/sciimmunol.aat1604.

Lomask, M. 2006. "Further Exploration of the Penh Parameter." *Experimental and Toxicologic Pathology* 57 (suppl. 2): 13–20. https://doi.org/10.1016/j.etp.2006.02.014.

Lommatzsch, M., P. Julius, M. Kuepper, H. Garn, K. Bratke, S. Irmscher, W. Luttmann, H. Renz, A. Braun, and J. C. Virchow. 2006. "The Course of Allergen-Induced Leukocyte Infiltration in Human and Experimental Asthma." *Journal of Allergy and Clinical Immunology* 118 (1): 91–97. https://doi.org/10.1016/j.jaci.2006.02.034.

Ludbrook, V. J., K. E. Hanrott, J. Marks-Konczalik, J. L. Kreindler, N. P. Bird, D. Hewens, M. Beerahee, et al. 2019. "A Placebo-Controlled, Double-Blind, Randomised, Crossover Study to Assess the Efficacy, Safety and Tolerability of TRPV4 Inhibitor GSK2798745 in Participants with Chronic Cough." *Thorax* 74 (Suppl 2): S27. https://doi.org/10.1136/thorax-2019-btsabstracts2019.33.

Lulich, K. M., and J. W. Paterson. 1980. "An in Vitro Study of Various Drugs on Central and Peripheral Airways of the Rat: A Comparison with Human Airways." *British Journal of Pharmacology* 68 (4): 633–36. https://doi.org/10.1111/j.1476-5381.1980.tb10854.x.

Lundblad, L. K. A., C. G. Irvin, Z. Hantos, P. Sly, W. Mitzner, and J. H. T. Bates. 2007. "Penh Is Not a Measure of Airway Resistance!" *European Respiratory Journal* 30 (4): 805. https://doi.org/10.1183/09031936.00091307.

Ma, B., H. Z. Ruan, G. Burnstock, and P. M. Dunn. 2005. "Differential Expression of P2X Receptors on Neurons from Different Parasympathetic Ganglia." *Neuropharmacology* 48

(5): 766–77. https://doi.org/10.1016/j.neuropharm.2004.12.021.

Ma, J. N., and E. S. Burstein. 2013. “The Protease Activated Receptor 2 (PAR2) Polymorphic Variant F240S Constitutively Activates Par2 Receptors and Potentiates Responses to Small-Molecule PAR2 Agonists.” *Journal of Pharmacology and Experimental Therapeutics* 347 (3): 697–704. https://doi.org/10.1124/jpet.113.208744.

Macfarlane, A. J., O. M. Kon, S. J. Smith, K. Zeibecoglou, L. N. Khan, L. T. Barata, A. R. McEuen, et al. 2000. “Basophils, Eosinophils, and Mast Cells in Atopic and Nonatopic Asthma and in Late-Phase Allergic Reactions in the Lung and Skin.” *Journal of Allergy and Clinical Immunology* 105 (1 I): 99–107. https://doi.org/10.1016/S0091-6749(00)90184-2.

MacGlashan, D. W., R. P. Schleimer, S. P. Peters, E. S. Schulman, G. K. Adams, H. H. Newball, and L. M. Lichtenstein. 1982. “Generation of Leukotrienes by Purified Human Lung Mast Cells.” *Journal of Clinical Investigation* 70 (4): 747–51. https://doi.org/10.1172/JCI110670.

Maher, S. A., M. A. Birrell, J. J. Adcock, M. A. Wortley, E. D. Dubuis, S. J. Bonvini, M. S. Grace, and M. G. Belvisi. 2015. “Prostaglandin D2 and the Role of the DP1, DP2 and TP Receptors in the Control of Airway Reflex Events.” *European Respiratory Journal* 45 (4): 1108–18. https://doi.org/10.1183/09031936.00061614.

Majhi, R. K., S. S. Sahoo, M. Yadav, B. M. Pratheek, S. Chattopadhyay, and C. Goswami. 2015. “Functional Expression of TRPV Channels in T Cells and Their Implications in Immune Regulation.” *FEBS Journal* 282 (14): 2661–81. https://doi.org/10.1111/febs.13306.

Manthei, D. M., D. J. Jackson, M. D. Evans, R. E. Gangnon, C. J. Tisler, J. E. Gern, R. F. Lemanske, and L. C. Denlinger. 2012. “Protection from Asthma in a High-Risk Birth Cohort by Attenuated P2X 7 Function.” *Journal of Allergy and Clinical Immunology* 130 (2): 496–502. https://doi.org/10.1016/j.jaci.2012.05.040.

Maric, Jovana, Avinash Ravindran, Luca Mazzurana, Aline Van Acker, Anna Rao, Efthymia Kokkinou, Maria Ekoff, et al. 2019. “Cytokine-Induced Endogenous Production of Prostaglandin D2 Is Essential for Human Group 2 Innate Lymphoid Cell Activation.” *Journal of Allergy and Clinical Immunology* 143 (6): 2202-2214.e5. https://doi.org/10.1016/j.jaci.2018.10.069.

Marone, G., F. Granata, V. Pucino, A. Pecoraro, E. Heffler, S. Loffredo, G. W. Scadding, and G. Varricchi. 2019. “The Intriguing Role of Interleukin 13 in the Pathophysiology of Asthma.” *Frontiers in Pharmacology* 10: 1387. https://doi.org/10.3389/fphar.2019.01387.

Martin, C., S. Uhlig, and V. Ullrich. 1996. “Videomicroscopy of Methacholine-Induced Contraction of Individual Airways in Precision-Cut Lung Slices.” *European Respiratory Journal* 9 (12): 2479–87. https://doi.org/10.1183/09031936.96.09122479.

Martin, T. R., N. P. Gerard, S. J. Galli, and J. M. Drazen. 1988. “Pulmonary Responses to Bronchoconstrictor Agonists in the Mouse.” *Journal of Applied Physiology* 64 (6): 2318–23. https://doi.org/10.1152/jappl.1988.64.6.2318.

Mazzone, S. B., and B. J. Canning. 2002a. “Evidence for Differential Reflex Regulation of Cholinergic and Noncholinergic Parasympathetic Nerves Innervating the Airways.” *American Journal of Respiratory and Critical Care Medicine* 165 (8): 1076–83. https://doi.org/10.1164/ajrccm.165.8.2001121270c.

———. 2002b. “Synergistic Interactions between Airway Afferent Nerve Subtypes Mediating Reflex Bronchospasm in Guinea Pigs.” *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 283 (1): R86-98.

https://doi.org/10.1152/ajpregu.00007.2002.

Mazzone, S. B., and B. J. Undem. 2016. “Vagal Afferent Innervation of the Airways in Health and Disease.” *Physiological Reviews* 96 (3): 975–1024. https://doi.org/10.1152/physrev.00039.2015.

McAlexander, M. A., M. A. Luttmann, G. E. Hunsberger, and B. J. Undem. 2014. “Transient Receptor Potential Vanilloid 4 Activation Constricts the Human Bronchus via the Release of Cysteinyl Leukotrienes.” *The Journal of Pharmacology and Experimental Therapeutics* 349 (1): 118–25. https://doi.org/10.1124/jpet.113.210203.

McGuire, J. J., M. Saifeddine, C. R. Triggle, K. Sun, and M. D. Hollenberg. 2004. “2-Furoyl-LIGRLO-Amide: A Potent and Selective Proteinase-Activated Receptor 2 Agonist.” *Journal of Pharmacology and Experimental Therapeutics* 309 (3): 1124–31. https://doi.org/10.1124/jpet.103.064584.

Mercer, R. R., M. L. Russell, V. L. Roggli, and J. D. Crapo. 1994. “Cell Number and Distribution in Human and Rat Airways.” *American Journal of Respiratory Cell and Molecular Biology* 10 (6): 613–24. https://doi.org/10.1165/ajrcmb.10.6.8003339.

Meyerholz, D. K., C. J. Suarez, S. M. Dintzis, and C. W. Frevert. 2018. “Respiratory System.” In *Comparative Anatomy and Histology*, 147–62. Elsevier. https://doi.org/10.1016/B978-0-12-802900-8.00009-9.

Meyts, I., J. A. Vanoirbeek, G. Hens, B. M. Vanaudenaerde, B. Verbinnen, D. M. A. Bullens, L. Overbergh, C. Mathieu, J. L. Ceuppens, and P. W. Hellings. 2008. “T-Cell Mediated Late Increase in Bronchial Tone after Allergen Provocation in a Murine Asthma Model.” *Clinical Immunology* 128 (2): 248–58. https://doi.org/10.1016/j.clim.2008.03.515.

Mihara, H., N. Suzuki, A. A. Boudaka, J. S. Muhammad, M. Tominaga, Y. Tabuchi, and T. Sugiyama. 2016. “Transient Receptor Potential Vanilloid 4-Dependent Calcium Influx and ATP Release in Mouse and Rat Gastric Epithelia.” *World Journal of Gastroenterology* 22 (24): 5512–19. https://doi.org/10.3748/wjg.v22.i24.5512.

Miller, H. R. P., and A. D. Pemberton. 2002. “Tissue-Specific Expression of Mast Cell Granule Serine Proteinases and Their Role in Inflammation in the Lung and Gut.” *Immunology* 105 (4): 375–90. https://doi.org/10.1046/j.1365-2567.2002.01375.x.

Minette, P. A. H., J. W. J. Lammers, C. M. S. Dixon, M. T. McCusker, and P. J. Barnes. 1989. “A Muscarinic Agonist Inhibits Reflex Bronchoconstriction in Normal but Not in Asthmatic Subjects.” *Journal of Applied Physiology* 67 (6): 2461–65. https://doi.org/10.1152/jappl.1989.67.6.2461.

Minshall, E., C. G. Wang, R. Dandurand, and D. Eidelman. 1997. “Heterogeneity of Responsiveness of Individual Airways in Cultured Lung Explants.” *Canadian Journal of Physiology and Pharmacology* 75 (7): 911–16. https://doi.org/10.1139/y97-107.

Mitchell, R. A., D. A. Herbert, and D. G. Baker. 1985. “Inspiratory Rhythm in Airway Smooth Muscle Tone.” *Journal of Applied Physiology* 58 (3): 911–20. https://doi.org/10.1152/jappl.1985.58.3.911.

Mizutani, N., H. Goshima, T. Nabe, and S. Yoshino. 2012. “Establishment and Characterization of a Murine Model for Allergic Asthma Using Allergen-Specific IgE Monoclonal Antibody to Study Pathological Roles of IgE.” *Immunology Letters* 141 (2): 235–45. https://doi.org/10.1016/j.imlet.2011.10.010.

Mochizuki, T., T. Sokabe, I. Araki, K. Fujishita, K. Shibasaki, K. Uchida, K. Naruse, S. Koizumi, M. Takeda, and M. Tominaga. 2009. “The TRPV4 Cation Channel Mediates Stretch-Evoked Ca2+ Influx and ATP Release in Primary Urothelial Cell Cultures.” *Journal of*

Biological Chemistry 284 (32): 21257–64. https://doi.org/10.1074/jbc.M109.020206.

Molet, S., D. Ramos-Barbón, J. G. Martin, and Q. Hamid. 1999. “Adoptively Transferred Late Allergic Response Is Inhibited by IL-4, but Not IL-5, Antisense Oligonucleotide.” *Journal of Allergy and Clinical Immunology* 104 (1): 205–14. https://doi.org/10.1016/S0091-6749(99)70137-5.

Molfino, N. A., A. S. Slutsky, G. Julià-Serdà, V. Hoffstein, J. P. Szalai, K. R. Chapman, A. S. Rebuck, and N. Zamel. 1993. “Assessment of Airway Tone in Asthma. Comparison between Double Lung Transplant Patients and Healthy Subjects.” *The American Review of Respiratory Disease* 148 (5): 1238–43. https://doi.org/10.1164/ajrccm/148.5.1238.

Mori, T., K. Saito, Y. Ohki, H. Arakawa, M. Tominaga, and K. Tokuyama. 2011. “Lack of Transient Receptor Potential Vanilloid-1 Enhances Th2-Biased Immune Response of the Airways in Mice Receiving Intranasal, but Not Intraperitoneal, Sensitization.” *International Archives of Allergy and Immunology* 156 (3): 305–12. https://doi.org/10.1159/000323889.

Mortola, J., G. Sant’ambrogio, and M. G. Clement. 1975. “Localization of Irritant Receptors in the Airways of the Dog.” *Respiration Physiology* 24 (1): 107–14. https://doi.org/10.1016/0034-5687(75)90125-5.

Moss, C. R., C. A. Gilbert, S. A. Gabriel, and Q. Gu. 2015. “Protease-Activated Receptor-2 Inhibits BK Channel Activity in Bronchopulmonary Sensory Neurons.” *Neuroscience Letters* 589: 13–18. https://doi.org/10.1016/j.neulet.2015.01.020.

Mounkaäila, B., R. Marthan, and E. Roux. 2005. “Biphasic Effect of Extracellular ATP on Human and Rat Airways Is Due to Multiple P2 Purinoceptor Activation.” *Respiratory Research* 6: 143. https://doi.org/10.1186/1465-9921-6-143.

Muccitelli, R. M., S. S. Tucker, D. W. P. Hay, T. J. Torphy, and M. A. Wasserman. 1987. “Is the Guinea Pig Trachea a Good in Vitro Model of Human Large and Central Airways? Comparison on Leukotriene-, Methacholine-, Histamine- and Antigen-Induced Contractions.” *Journal of Pharmacology and Experimental Therapeutics* 243 (2): 467–73.

Müller, T., R. P. Vieira, M. Grimm, T. Dürk, S. Cicko, R. Zeiser, T. Jakob, et al. 2011. “A Potential Role for P2X7R in Allergic Airway Inflammation in Mice and Humans.” *American Journal of Respiratory Cell and Molecular Biology* 44 (4): 456–64. https://doi.org/10.1165/rcmb.2010-0129OC.

Nabe, T., K. Matsuya, K. Akamizu, M. Fujita, T. Nakagawa, M. Shioe, H. Kida, et al. 2013. “Roles of Basophils and Mast Cells Infiltrating the Lung by Multiple Antigen Challenges in Asthmatic Responses of Mice.” *British Journal of Pharmacology* 169 (2): 462–76. https://doi.org/10.1111/bph.12154.

Nabe, T., T. Morishita, K. Matsuya, A. Ikedo, M. Fujii, N. Mizutani, and S. Yoshino. 2011. “Complete Dependence on CD4 + Cells in Late Asthmatic Response, but Limited Contribution of the Cells to Airway Remodeling in Sensitized Mice.” *Journal of Pharmacological Sciences* 116 (4): 373–83. https://doi.org/10.1254/jphs.11083FP.

Nabe, T., C. L. Zindl, W. J. Yong, R. Stephens, A. Sakamoto, S. Kohno, T. P. Atkinson, and D. D. Chaplin. 2005. “Induction of a Late Asthmatic Response Associated with Airway Inflammation in Mice.” *European Journal of Pharmacology* 521 (1–3): 144–55. https://doi.org/10.1016/j.ejphar.2005.08.015.

Naert, R., A. López-Requena, T. Voets, K. Talavera, and Y. A. Alpizar. 2019. “Expression and Functional Role of TRPV4 in Bone Marrow-Derived CD11C+ Cells.” *International Journal of Molecular Sciences* 20 (14). https://doi.org/10.3390/ijms20143378.

Nagaoka, M., M. Nara, T. Tamada, H. Kume, T. Oguma, T. Kikuchi, J. Zaini, et al. 2009.

"Regulation of Adenosine 5′-Triphosphate (ATP)-Gated P2X4 Receptors on Tracheal Smooth Muscle Cells." *Respiratory Physiology and Neurobiology* 166 (1): 61–67. https://doi.org/10.1016/j.resp.2009.02.002.

Nakaya, M., M. Dohi, K. Okunishi, K. Nakagome, R. Tanaka, M. Imamura, S. Baba, N. Takeuchi, K. Yamamoto, and K. Kaga. 2006. "Noninvasive System for Evaluating Allergen-Induced Nasal Hypersensitivity in Murine Allergic Rhinitis." *Laboratory Investigation* 86 (9): 917–26. https://doi.org/10.1038/labinvest.3700452.

Nasra, J., and M. G. Belvisi. 2009. "Modulation of Sensory Nerve Function and the Cough Reflex: Understanding Disease Pathogenesis." *Pharmacology and Therapeutics* 124 (3): 354–75. https://doi.org/10.1016/j.pharmthera.2009.09.006.

Nassenstein, C., K. Kwong, T. Taylor-Clark, M. Kollarik, D. M. Macglashan, A. Braun, and B. J. Undem. 2008. "Expression and Function of the Ion Channel TRPA1 in Vagal Afferent Nerves Innervating Mouse Lungs." *Journal of Physiology* 586 (6): 1595–1604. https://doi.org/10.1113/jphysiol.2007.148379.

Nassenstein, C., T. E. Taylor-Clark, A. C. Myers, F. Ru, R. Nandigama, W. Bettner, and B. J. Undem. 2010. "Phenotypic Distinctions between Neural Crest and Placodal Derived Vagal C-Fibres in Mouse Lungs." *Journal of Physiology* 588 (23): 4769–83. https://doi.org/10.1113/jphysiol.2010.195339.

Neuhaus, V., D. Schaudien, T. Golovina, U. A. Temann, C. Thompson, T. Lippmann, C. Bersch, et al. 2017. "Assessment of Long-Term Cultivated Human Precision-Cut Lung Slices as an Ex Vivo System for Evaluation of Chronic Cytotoxicity and Functionality." *Journal of Occupational Medicine and Toxicology* 12 (1): 13. https://doi.org/10.1186/s12995-017-0158-5.

Ni, D., Q. Gu, H. Z. Hu, N. Gao, M. X. Zhu, and L. Y. Lee. 2006. "Thermal Sensitivity of Isolated Vagal Pulmonary Sensory Neurons: Role of Transient Receptor Potential Vanilloid Receptors." *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 291 (3): R541-50. https://doi.org/10.1152/ajpregu.00016.2006.

Nichols, H. L., M. Saffeddine, B. S. Theriot, A. Hegde, D. Polley, T. El-Mays, H. Vliagoftis, et al. 2012. "β-Arrestin-2 Mediates the Proinflammatory Effects of Proteinase-Activated Receptor-2 in the Airway." *Proceedings of the National Academy of Sciences of the United States of America* 109 (41): 16660–65. https://doi.org/10.1073/pnas.1208881109.

Nieber, K., C. R. Baumgarten, R. Rathsack, J. Furkert, P. Oehme, and G. Kunkel. 1992. "Substance P and β-Endorphin-like Immunoreactivity in Lavage Fluids of Subjects with and without Allergic Asthma." *The Journal of Allergy and Clinical Immunology* 90 (4 PART 1): 646–52. https://doi.org/10.1016/0091-6749(92)90138-R.

Nonaka, T., H. Mitsuhashi, K. Takahashi, H. Sugiyama, and T. Kishimoto. 2000. "Effect of TEI-9874, an Inhibitor of Immunoglobulin E Production, on Allergen-Induced Asthmatic Model in Rats." *European Journal of Pharmacology* 402 (3): 287–95. https://doi.org/10.1016/S0014-2999(00)00530-6.

North, R. A. 2002. "Molecular Physiology of P2X Receptors." *Physiological Reviews* 82 (4): 1013–67. https://doi.org/10.1152/physrev.00015.2002.

O'Byrne, P. M. 2009. "Allergen-Induced Airway Inflammation and Its Therapeutic Intervention." *Allergy, Asthma and Immunology Research* 1 (1): 3–9. https://doi.org/10.4168/aair.2009.1.1.3.

O'Byrne, P. M., G. M. Gauvreau, and J. D. Brannan. 2009. "Provoked Models of Asthma: What Have We Learnt?" *Clinical and Experimental Allergy* 39 (2): 181–92. https://doi.org/10.1111/j.1365-2222.2008.03172.x.

O'Sullivan, S., A. Roquet, B. Dahlén, S. E. Dahlén, and M. Kumlin. 1998. "Urinary Excretion of Inflammatory Mediators during Allergen-Induced Early and Late Phase Asthmatic Reactions." *Clinical and Experimental Allergy* 28 (11): 1332–39. https://doi.org/10.1046/j.1365-2222.1998.00368.x.

Ohta, S., N. Oda, T. Yokoe, A. Tanaka, Y. Yamamoto, Y. Watanabe, K. Minoguchi, et al. 2010. "Effect of Tiotropium Bromide on Airway Inflammation and Remodelling in a Mouse Model of Asthma." *Clinical and Experimental Allergy* 40 (8): 1266–75. https://doi.org/10.1111/j.1365-2222.2010.03478.x.

Ohtomo, T., O. Kaminuma, N. Kitamura, M. Suko, N. Kobayashi, and A. Mori. 2009. "Murine Th Clones Confer Late Asthmatic Response upon Antigen Challenge." *International Archives of Allergy and Immunology* 149 (Suppl 1): 2–6. https://doi.org/10.1159/000210646.

Paggiaro, P., D. M. G. Halpin, R. Buhl, M. Engel, V. B. Zubek, Z. Blahova, P. Moroni-Zentgraf, and E. Pizzichini. 2016. "The Effect of Tiotropium in Symptomatic Asthma Despite Low- to Medium-Dose Inhaled Corticosteroids: A Randomized Controlled Trial." *Journal of Allergy and Clinical Immunology: In Practice* 4 (1): 104-113.e2. https://doi.org/10.1016/j.jaip.2015.08.017.

Palaniyandi, S., A. M. Rajendrakumar, S. Periasamy, R. Goswami, W. Tuo, X. Zhu, and S. O. Rahaman. 2019. "TRPV4 Is Dispensable for the Development of Airway Allergic Asthma." *Laboratory Investigation*. https://doi.org/10.1038/s41374-019-0305-y.

Parameswaran, K., M. D. Inman, R. M. Watson, M. M. Morris, A. Efthimiadis, P. G. Ventresca, R. Lam, P. M. O'Byrne, and F. E. Hargreave. 2000. "Protective Effects of Fluticasone on Allergen-Induced Airway Responses and Sputum Inflammatory Markers." *Canadian Respiratory Journal* 7 (4): 313–19. https://doi.org/10.1155/2000/254213.

Patel, H. J., P. J. Barnes, T. Takahashi, S. Tadjkarimi, M. H. Yacoub, and M. G. Belvisi. 1995. "Evidence for Prejunctional Muscarinic Autoreceptors in Human and Guinea Pig Trachea." *American Journal of Respiratory and Critical Care Medicine* 152 (3): 872–78. https://doi.org/10.1164/ajrccm.152.3.7663798.

Pavord, I. D., R. Beasley, A. Agusti, G. P. Anderson, E. Bel, G. Brusselle, P. Cullinan, et al. 2018. "After Asthma: Redefining Airways Diseases." *The Lancet* 391 (10118): 350–400. https://doi.org/10.1016/S0140-6736(17)30879-6.

Pedersen, B., R. Dahl, B. B. Larsen, and P. Venge. 1993. "The Effect of Salmeterol on the Early- and Late-phase Reaction to Bronchial Allergen and Postchallenge Variation in Bronchial Reactivity, Blood Eosinophils, Serum Eosinophil Cationic Protein, and Serum Eosinophil Protein X." *Allergy* 48 (5): 377–82. https://doi.org/10.1111/j.1398-9995.1993.tb02410.x.

Pedersen, S. F., G. Owsianik, and B. Nilius. 2005. "TRP Channels: An Overview." *Cell Calcium* 38: 233–52. https://doi.org/10.1016/j.ceca.2005.06.028.

Pejler, G. 2019. "The Emerging Role of Mast Cell Proteases in Asthma." *The European Respiratory Journal* 54 (4): 1900685. https://doi.org/10.1183/13993003.00685-2019.

Pelleg, A., F. Xu, J. Zhuang, B. Undem, and G. Burnstock. 2019. "DT-0111: A Novel Drug-Candidate for the Treatment of COPD and Chronic Cough." *Therapeutic Advances in Respiratory Disease* 13: 175346661987796. https://doi.org/10.1177/1753466619877960.

Pennock, N. D., J. T. White, E. W. Cross, E. E. Cheney, B. A. Tamburini, and R. M. Kedl. 2013. "T Cell Responses: Naïve to Memory and Everything in Between." *American Journal of Physiology - Advances in Physiology Education* 37 (4): 273–83.

https://doi.org/10.1152/advan.00066.2013.

Perez, J. F., and M. J. Sanderson. 2005. “The Frequency of Calcium Oscillations Induced by 5-HT, ACH, and KCl Determine the Contraction of Smooth Muscle Cells of Intrapulmonary Bronchioles.” *The Journal of General Physiology* 125 (6): 535–53. https://doi.org/10.1085/jgp.200409216.

Perkins, C., M. Wills-Karp, and F. D. Finkelman. 2006. “IL-4 Induces IL-13-Independent Allergic Airway Inflammation.” *Journal of Allergy and Clinical Immunology* 118 (2): 410–19. https://doi.org/10.1016/j.jaci.2006.06.004.

Peters, S. P., S. J. Kunselman, N. Icitovic, W. C. Moore, R. Pascual, B. T. Ameredes, H. A. Boushey, et al. 2010. “Tiotropium Bromide Step-up Therapy for Adults with Uncontrolled Asthma.” *New England Journal of Medicine* 363 (18): 1715–26. https://doi.org/10.1056/NEJMoa1008770.

Phalen, R. F., and M. J. Oldham. 1983. “Tracheobronchial Airway Structure as Revealed by Casting Techniques.” *American Review of Respiratory Disease* 128 (2 Pt 2): S1-4. https://doi.org/10.1164/arrd.1983.128.2P2.S1.

Pizzichini, M. M. M., J. C. Kidney, B. J. O. Wong, M. M. Morris, A. Efthimiadis, J. Dolovich, and F. E. Hargreave. 1996. “Effect of Salmeterol Compared with Beclomethasone on Allergen-Induced Asthmatic and Inflammatory Responses.” *European Respiratory Journal* 9 (3): 449–55. https://doi.org/10.1183/09031936.96.09030449.

Poole, D. P., S. Amadesi, N. A. Veldhuis, F. C. Abogadie, T. Lieu, W. Darby, W. Liedtke, M. J. Lew, P. McIntyre, and N. W. Bunnett. 2013. “Protease-Activated Receptor 2 (PAR2) Protein and Transient Receptor Potential Vanilloid 4 (TRPV4) Protein Coupling Is Required for Sustained Inflammatory Signaling.” *The Journal of Biological Chemistry* 288 (8): 5790–5802. https://doi.org/10.1074/jbc.M112.438184.

Postma, D. S., C. Brightling, S. Baldi, M. Van Den Berge, L. M. Fabbri, A. Gagnatelli, A. Papi, et al. 2019. “Exploring the Relevance and Extent of Small Airways Dysfunction in Asthma (ATLANTIS): Baseline Data from a Prospective Cohort Study.” *The Lancet Respiratory Medicine* 7 (5): 402–16. https://doi.org/10.1016/S2213-2600(19)30049-9.

Potenzieri, C., S. Meeker, and B. J. Undem. 2012. “Activation of Mouse Bronchopulmonary C-Fibres by Serotonin and Allergen-Ovalbumin Challenge.” *The Journal of Physiology* 590 (21): 5449–59. https://doi.org/10.1113/jphysiol.2012.237115.

Price, D., M. Fletcher, and T. Van Der Molen. 2014. “Asthma Control and Management in 8,000 European Patients: The REcognise Asthma and LInk to Symptoms and Experience (REALISE) Survey.” *Npj Primary Care Respiratory Medicine* 24. https://doi.org/10.1038/npjpcrm.2014.9.

Rabe, K. F., P. Nair, G. Brusselle, J. F. Maspero, M. Castro, L. Sher, H. Zhu, et al. 2018. “Efficacy and Safety of Dupilumab in Glucocorticoid-Dependent Severe Asthma.” *New England Journal of Medicine* 378 (26): 2475–85. https://doi.org/10.1056/NEJMoa1804093.

Raemdonck, K., J. de Alba, M. A. Birrell, M. Grace, S. A. Maher, C. G. Irvin, J. R. Fozard, P. M. O’Byrne, and M. G. Belvisi. 2012. “A Role for Sensory Nerves in the Late Asthmatic Response.” *Thorax* 67 (1): 19–25. https://doi.org/10.1136/thoraxjnl-2011-200365.

Rahaman, S. O., L. M. Grove, S. Paruchuri, B. D. Southern, S. Abraham, K. A. Niese, R. G. Scheraga, et al. 2014. “TRPV4 Mediates Myofibroblast Differentiation and Pulmonary Fibrosis in Mice.” *Journal of Clinical Investigation* 124 (12): 5225–38. https://doi.org/10.1172/JCI75331.

Rahman, M., R. Sun, S. Mukherjee, B. Nilius, and L. J. Janssen. 2018. “TRPV4 Stimulation Releases ATP via Pannexin Channels in Human Pulmonary Fibroblasts.” *American Journal of Respiratory Cell and Molecular Biology* 59 (1): 87–95. https://doi.org/10.1165/rcmb.2017-0413OC.

Rajasekhar, P., D. P. Poole, W. Liedtke, N. W. Bunnett, and N. A. Veldhuis. 2015. “P2Y1 Receptor Activation of the TRPV4 Ion Channel Enhances Purinergic Signaling in Satellite Glial Cells.” *Journal of Biological Chemistry* 290 (48): 29051–62. https://doi.org/10.1074/jbc.M115.689729.

Rehman, R., Y. A. Bhat, L. Panda, and U. Mabalirajan. 2013. “TRPV1 Inhibition Attenuates IL-13 Mediated Asthma Features in Mice by Reducing Airway Epithelial Injury.” *International Immunopharmacology* 15 (3): 597–605. https://doi.org/10.1016/j.intimp.2013.02.010.

Ressmeyer, A. R., A. K. Larsson, E. Vollmer, S. E. Dahlèn, S. Uhlig, and C. Martin. 2006. “Characterisation of Guinea Pig Precision-Cut Lung Slices: Comparison with Human Tissues.” *European Respiratory Journal* 28 (3): 603–11. https://doi.org/10.1183/09031936.06.00004206.

Ricciardolo, F. L. M., M. Steinhoff, S. Amadesi, R. Guerrini, M. Tognetto, M. Trevisani, C. Creminon, et al. 2000. “Presence and Bronchomotor Activity of Protease-Activated Receptor-2 in Guinea Pig Airways.” *American Journal of Respiratory and Critical Care Medicine* 161 (5): 1672–80. https://doi.org/10.1164/ajrccm.161.5.9907133.

Riccio, M. M., A. C. Myers, and B. J. Undem. 1996. “Immunomodulation of Afferent Neurons in Guinea-Pig Isolated Airway.” *Journal of Physiology* 491 (2): 499–509. https://doi.org/10.1113/jphysiol.1996.sp021234.

Ricco, M. M., W. Kummer, B. Biglari, A. C. Myers, and B. J. Undem. 1996. “Interganglionic Segregation of Distinct Vagal Afferent Fibre Phenotypes in Guinea-Pig Airways.” *The Journal of Physiology* 496 (2): 521–30. https://doi.org/10.1113/jphysiol.1996.sp021703.

Richardson, J., and J. Beland. 1976. “Nonadrenergic Inhibitory Nervous System in Human Airways.” *Journal of Applied Physiology* 41 (5 (I)): 764–71. https://doi.org/10.1152/jappl.1976.41.5.764.

Riley, J. P., B. Fuchs, L. Sjöberg, G. P. Nilsson, L. Karlsson, S. E. Dahlén, N. L. Rao, and M. Adner. 2013. “Mast Cell Mediators Cause Early Allergic Bronchoconstriction in Guinea-Pigs in Vivo: A Model of Relevance to Asthma.” *Clinical Science* 125 (11): 533–42. https://doi.org/10.1042/CS20130092.

Robinson, D., Q. Hamid, A. Bentley, S. Ying, A. B. Kay, and S. R. Durham. 1993. “Activation of CD4+ T Cells, Increased TH2-Type Cytokine MRNA Expression, and Eosinophil Recruitment in Bronchoalveolar Lavage after Allergen Inhalation Challenge in Patients with Atopic Asthma.” *The Journal of Allergy and Clinical Immunology* 92 (2): 313–24. https://doi.org/10.1016/0091-6749(93)90175-F.

Robinson, D. S. 2014. “Inhaled Allergen Challenge in Assessment of Biologics for Asthma.” *Clinical & Experimental Allergy* 44 (1): 2–5. https://doi.org/10.1111/cea.12234.

Robinson, R. K., M. A. Birrell, J. J. Adcock, M. A. Wortley, E. D. Dubuis, S. Chen, C. M. McGilvery, et al. 2018. “Mechanistic Link between Diesel Exhaust Particles and Respiratory Reflexes.” *Journal of Allergy and Clinical Immunology* 141 (3): 1074-1084.e9. https://doi.org/10.1016/j.jaci.2017.04.038.

Rogers, D. F. 2001. “Motor Control of Airway Goblet Cells and Glands.” *Respiration Physiology* 125 (1–2): 129–44. https://doi.org/10.1016/s0034-5687(00)00209-7.

Roquet, A., B. Dahlén, M. Kumlin, E. Ihre, G. Anstrén, S. Binks, and S. E. Dahlén. 1997. "Combined Antagonism of Leukotrienes and Histamine Produces Predominant Inhibition of Allergen-Induced Early and Late Phase Airway Obstruction in Asthmatics." *American Journal of Respiratory and Critical Care Medicine* 155 (6): 1856–63. https://doi.org/10.1164/ajrccm.155.6.9196086.

Rosenthal, R. R., P. S. Norman, W. R. Summer, and S. Permutt. 1977. "Role of the Parasympathetic System in Antigen Induced Bronchospasm." *J. Appl. Physiol. Respir. Environ. Exercise Physiol.* 42 (4): 600–606. https://doi.org/10.1152/jappl.1977.42.4.600.

Ruffin, R. E., D. W. Cockcroft, and F. E. Hargreave. 1978. "A Comparison of the Protective Effect of Fenoterol and Sch1000 on Allergen-Induced Asthma." *The Journal of Allergy and Clinical Immunology* 61 (1): 42–47. https://doi.org/10.1016/0091-6749(78)90472-4.

Sanderson, M. J., P. Delmotte, Y. Bai, and J. F. Perez-Zogbhi. 2008. "Regulation of Airway Smooth Muscle Cell Contractility by Ca2+ Signaling and Sensitivity." *Proceedings of the American Thoracic Society* 5 (1): 23–31. https://doi.org/10.1513/pats.200704-050VS.

Sant'Ambrogio, G., J. E. Remmers, W. J. De Groot, G. Callas, and J. P. Mortola. 1978. "Localization of Rapidly Adapting Receptors in the Trachea and Main Stem Bronchus of the Dog." *Respiration Physiology* 33 (3): 359–66. https://doi.org/10.1016/0034-5687(78)90062-2.

Satia, I., N. Tsamandouras, K. Holt, H. Badri, M. Woodhead, K. Ogungbenro, T. W. Felton, P. M. O'Byrne, S. J. Fowler, and J. A. Smith. 2017. "Capsaicin-Evoked Cough Responses in Asthmatic Patients: Evidence for Airway Neuronal Dysfunction." *Journal of Allergy and Clinical Immunology* 139 (3): 771-779.e10. https://doi.org/10.1016/j.jaci.2016.04.045.

Satia, I., R. Watson, T. Scime, R. J. Dockry, S. Sen, J. W. Ford, P. D. Mitchell, et al. 2019. "Allergen Challenge Increases Capsaicin-Evoked Cough Responses in Patients with Allergic Asthma." *Journal of Allergy and Clinical Immunology* 144 (3): 788-795.e1. https://doi.org/10.1016/j.jaci.2018.11.050.

Scheerens, H., J. R. Arron, Y. Zheng, W. S. Putnam, R. W. Erickson, D. F. Choy, J. M. Harris, J. Lee, N. N. Jarjour, and J. G. Matthews. 2014. "The Effects of Lebrikizumab in Patients with Mild Asthma Following Whole Lung Allergen Challenge." *Clinical & Experimental Allergy* 44 (1): 38–46. https://doi.org/10.1111/cea.12220.

Schelegle, E. S., and J. F. Green. 2001. "An Overview of the Anatomy and Physiology of Slowly Adapting Pulmonary Stretch Receptors." *Respiration Physiology* 125 (1–2): 17–31. https://doi.org/10.1016/S0034-5687(00)00202-4.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, et al. 2012. "Fiji: An Open-Source Platform for Biological-Image Analysis." *Nature Methods* 9 (7): 676–82. https://doi.org/10.1038/nmeth.2019.

Schmidlin, F., S. Amadesi, K. Dabbagh, D. E. Lewis, P. Knott, N. W. Bunnett, P. R. Gater, P. Geppetti, C. Bertrand, and M. E. Stevens. 2002. "Protease-Activated Receptor 2 Mediates Eosinophil Infiltration and Hyperreactivity in Allergic Inflammation of the Airway." *The Journal of Immunology* 169 (9): 5315–21. https://doi.org/10.4049/jimmunol.169.9.5315.

Schwartz, L. B., A. M. Irani, K. Roller, M. C. Castells, and N. M. Schechter. 1987. "Quantitation of Histamine, Tryptase, and Chymase in Dispersed Human T and TC Mast Cells." *Journal of Immunology (Baltimore, Md.: 1950)* 138 (8): 2611–15. http://www.ncbi.nlm.nih.gov/pubmed/3549903.

Sedgwick, J. B., W. J. Calhoun, G. J. Gleich, H. Kita, J. S. Abrams, L. B. Schwartz, B. Volovitz, M. Ben-Yaakov, and W. W. Busse. 1991. "Immediate and Late Airway Response of

Allergic Rhinitis Patients to Segmental Antigen Challenge: Characterization of Eosinophil and Mast Cell Mediators." *American Review of Respiratory Disease* 144 (6): 1274–81. https://doi.org/10.1164/ajrccm/144.6.1274.

Seehase, S., M. Schlepütz, S. Switalla, K. Mätz-Rensing, F. J. Kaup, M. Zöller, C. Schlumbohm, et al. 2011. "Bronchoconstriction in Nonhuman Primates: A Species Comparison." *Journal of Applied Physiology* 111 (3): 791–98. https://doi.org/10.1152/japplphysiol.00162.2011.

Seminario-Vidal, L., S. F. Okada, J. I. Sesma, S. M. Kreda, C. A. Van Heusden, Y. Zhu, L. C. Jones, et al. 2011. "Rho Signaling Regulates Pannexin 1-Mediated ATP Release from Airway Epithelia." *Journal of Biological Chemistry* 286 (30): 26277–86. https://doi.org/10.1074/jbc.M111.260562.

Sevigny, L. M., P. Zhang, A. Bohm, K. Lazarides, G. Perides, L. Covic, and A. Kuliopulos. 2011. "Interdicting Protease-Activated Receptor-2-Driven Inflammation with Cell-Penetrating Pepducins." *Proceedings of the National Academy of Sciences of the United States of America* 108 (20): 8491–96. https://doi.org/10.1073/pnas.1017091108.

Shamji, Mohamed H., and Stephen R. Durham. 2017. "Mechanisms of Allergen Immunotherapy for Inhaled Allergens and Predictive Biomarkers." *Journal of Allergy and Clinical Immunology* 140 (6): 1485–98. https://doi.org/10.1016/j.jaci.2017.10.010.

Shearer, A. M., R. Rana, K. Austin, J. D. Baleja, N. Nguyen, A. Bohm, L. Covic, and A. Kuliopulos. 2016. "Targeting Liver Fibrosis with a Cell-Penetrating Protease-Activated Receptor-2 (PAR2) Pepducin." *Journal of Biological Chemistry* 291 (44): 23188–98. https://doi.org/10.1074/jbc.M116.732743.

Shibasaki, K., K. Ikenaka, F. Tamalu, M. Tominaga, and Y. Ishizaki. 2014. "A Novel Subtype of Astrocytes Expressing TRPV4 (Transient Receptor Potential Vanilloid 4) Regulates Neuronal Excitability via Release of Gliotransmitters." *The Journal of Biological Chemistry* 289 (21): 14470–80. https://doi.org/10.1074/jbc.M114.557132.

Silverman, W., S. Locovei, and G. Dahl. 2008. "Probenecid, a Gout Remedy, Inhibits Pannexin 1 Channels." *American Journal of Physiology - Cell Physiology* 295 (3). https://doi.org/10.1152/ajpcell.00227.2008.

Singh, D., P. Cadden, M. Hunter, L. P. Collins, M. Perkins, R. Pettipher, E. Townsend, and S. Vinall. 2013. "Inhibition of the Asthmatic Allergen Challenge Response by the CRTH2 Antagonist OC000459." *European Respiratory Journal* 41 (1): 46–52. https://doi.org/10.1183/09031936.00092111.

Sipe, W. E. B., S. M. Brierley, C. M. Martin, B. D. Phillis, F. B. Cruz, E. F. Grady, W. Liedtke, et al. 2008. "Transient Receptor Potential Vanilloid 4 Mediates Protease Activated Receptor 2-Induced Sensitization of Colonic Afferent Nerves and Visceral Hyperalgesia." *American Journal of Physiology. Gastrointestinal and Liver Physiology* 294 (5): G1288-98. https://doi.org/10.1152/ajpgi.00002.2008.

Sluyter, R., J. A. Barden, and J. S. Wiley. 2001. "Detection of P2X Purinergic Receptors on Human B Lymphocytes." *Cell and Tissue Research* 304 (2): 231–36. https://doi.org/10.1007/s004410100372.

Smit, M., A. B. Zuidhof, S. I.T. Bos, H. Maarsingh, R. Gosens, J. Zaagsma, and H. Meurs. 2014. "Bronchoprotection by Olodaterol Is Synergistically Enhanced by Tiotropium in a Guinea Pig Model of Allergic Asthma." *Journal of Pharmacology and Experimental Therapeutics* 348 (2): 303–10. https://doi.org/10.1124/jpet.113.208439.

Smith, Jaclyn A, Michael M Kitt, Alyn H Morice, Surinder S Birring, Lorcan P McGarvey, Mandel R Sher, Yu-Ping Li, et al. 2020. "Gefapixant, a P2X3 Receptor Antagonist, for the

Treatment of Refractory or Unexplained Chronic Cough: A Randomised, Double-Blind, Controlled, Parallel-Group, Phase 2b Trial." *The Lancet Respiratory Medicine* 8 (8): 775–85. https://doi.org/10.1016/s2213-2600(19)30471-0.

Spaulding, E. L., and R. W. Burgess. 2017. "Accumulating Evidence for Axonal Translation in Neuronal Homeostasis." *Frontiers in Neuroscience* 11. https://doi.org/10.3389/fnins.2017.00312.

Steinhoff, M., N. Vergnolle, S. H. Young, M. Tognetto, S. Amadesi, H. S. Ennes, M. Trevisani, et al. 2000. "Agonists of Proteinase-Activated Receptor 2 Induce Inflammation by a Neurogenic Mechanism." *Nature Medicine* 6 (2): 151–58. https://doi.org/10.1038/72247.

Steinke, J. W., and L. Borish. 2001. "Th2 Cytokines and Asthma. Interleukin-4: Its Role in the Pathogenesis of Asthma, and Targeting It for Asthma Treatment with Interleukin-4 Receptor Antagonists." *Respiratory Research* 2 (2): 66–70. https://doi.org/10.1186/rr40.

Stenberg, H., Z. Diamant, J. Ankerst, L. Bjermer, and E. Tufvesson. 2017. "Small Airway Involvement in the Late Allergic Response in Asthma." *Clinical and Experimental Allergy* 47 (12): 1555–65. https://doi.org/10.1111/cea.13036.

Stevenson, C. S., and M. G. Belvisi. 2008. "Preclinical Animal Models of Asthma and Chronic Obstructive Pulmonary Disease." *Expert Review of Respiratory Medicine* 2 (5): 631–43. https://doi.org/10.1586/17476348.2.5.631.

Stevenson, C. S., and M. A. Birrell. 2011. "Moving towards a New Generation of Animal Models for Asthma and COPD with Improved Clinical Relevance." *Pharmacology and Therapeutics* 130 (2): 93–105. https://doi.org/10.1016/j.pharmthera.2010.10.008.

Stokes Peebles, R., R. G. Hamilton, L. M. Lichtenstein, M. Schlosberg, M. C. Liu, D. Proud, and A. Togias. 2001. "Antigen-Specific IgE and IgA Antibodies in Bronchoalveolar Lavage Fluid Are Associated with Stronger Antigen-Induced Late Phase Reactions." *Clinical & Experimental Allergy* 31 (2): 239–48. https://doi.org/10.1046/j.1365-2222.2001.01048.x.

Strotmann, R., C. Harteneck, K. Nunnenmacher, G. Schultz, and T D. Plant. 2000. "OTRPC4, a Nonselective Cation Channel That Confers Sensitivity to Extracellular Osmolarity." *Nature Cell Biology* 2 (10): 695–702. https://doi.org/10.1038/35036318.

Suen, J. Y., A. Cotterell, R. J. Lohman, J. Lim, A. Han, M. K. Yau, L. Liu, M. A. Cooper, D. A. Vesey, and D. P. Fairlie. 2014. "Pathway-Selective Antagonism of Proteinase Activated Receptor 2." *British Journal of Pharmacology* 171 (17): 4112–24. https://doi.org/10.1111/bph.12757.

Swirski, F. K., B. U. Gajewska, D. Alvarez, S. A. Ritz, M. J. Cundall, E. C. Cates, A. J. Coyle, et al. 2002. "Inhalation of a Harmless Antigen (Ovalbumin) Elicits Immune Activation but Divergent Immunoglobulin and Cytokine Activities in Mice." *Clinical and Experimental Allergy* 32 (3): 411–21. https://doi.org/10.1046/j.1365-2222.2002.01291.x.

Szefler, S. J., B. R. Phillips, F. D. Martinez, V. M. Chinchilli, R. F. Lemanske, R. C. Strunk, R. S. Zeiger, et al. 2005. "Characterization of Within-Subject Responses to Fluticasone and Montelukast in Childhood Asthma." *Journal of Allergy and Clinical Immunology* 115 (2): 233–42. https://doi.org/10.1016/j.jaci.2004.11.014.

Talbot, S., R. E. E. Abdulnour, P. R. Burkett, S. Lee, S. J. F. Cronin, M. A. Pascal, C. Laedermann, et al. 2015. "Silencing Nociceptor Neurons Reduces Allergic Airway Inflammation." *Neuron* 87 (2): 341–54. https://doi.org/10.1016/j.neuron.2015.06.007.

Talbot, Sébastien, Simmie L. Foster, and Clifford J. Woolf. 2016. "Neuroimmunity: Physiology and Pathology." *Annual Review of Immunology* 34 (1): 421–47.

https://doi.org/10.1146/annurev-immunol-041015-055340.

Tatar, M., G. Sant'Ambrogio, and F. B. Sant'Ambrogio. 1994. "Laryngeal and Tracheobronchial Cough in Anesthetized Dogs." *Journal of Applied Physiology* 76 (6): 2672–79. https://doi.org/10.1152/jappl.1994.76.6.2672.

Taube, C., C. Duez, Z. H. Cui, K. Takeda, Y. H. Rha, J. W. Park, A. Balhorn, D. D. Donaldson, A. Dakhama, and E. W. Gelfand. 2002. "The Role of IL-13 in Established Allergic Airway Disease." *The Journal of Immunology* 169 (11): 6482–89. https://doi.org/10.4049/jimmunol.169.11.6482.

Taylor-Clark, T. E., F. Kiros, M. J. Carr, and M. A. McAlexander. 2009. "Transient Receptor Potential Ankyrin 1 Mediates Toluene Diisocyanate-Evoked Respiratory Irritation." *American Journal of Respiratory Cell and Molecular Biology* 40 (6): 756–62. https://doi.org/10.1165/rcmb.2008-0292OC.

Taylor-Clark, T. E., C. Nassenstein, and B. J. Undem. 2008. "Leukotriene D 4 Increases the Excitability of Capsaicin-Sensitive Nasal Sensory Nerves to Electrical and Chemical Stimuli." *British Journal of Pharmacology* 154 (6): 1359–68. https://doi.org/10.1038/bjp.2008.196.

Taylor-Clark, T. E., and B. J. Undem. 2010. "Ozone Activates Airway Nerves via the Selective Stimulation of TRPA1 Ion Channels." *The Journal of Physiology* 588 (3): 423–33. https://doi.org/10.1113/jphysiol.2009.183301.

Taylor, A. L., L. M. Schwiebert, J. J. Smith, C. King, J. R. Jones, E. J. Sorscher, and E. M. Schwiebert. 1999. "Epithelial P2X Purinergic Receptor Channel Expression and Function." *Journal of Clinical Investigation* 104 (7): 875–84. https://doi.org/10.1172/JCI7270.

Taylor, I. K., K. M. O'Shaughnessy, N. B. Choudry, M. Adachi, J. B. D. Palmer, and R. W. Fuller. 1992. "A Comparative Study in Atopic Subjects with Asthma of the Effects of Salmeterol and Salbutamol on Allergen-Induced Bronchoconstriction, Increase in Airway Reactivity, and Increase in Urinary Leukotriene E4 Excretion." *The Journal of Allergy and Clinical Immunology* 89 (2): 575–83. https://doi.org/10.1016/0091-6749(92)90325-V.

Temann, A., T. Golovina, V. Neuhaus, C. Thompson, J. A. Chichester, A. Braun, and V. Yusibov. 2017. "Evaluation of Inflammatory and Immune Responses in Long-Term Cultured Human Precision-Cut Lung Slices." *Human Vaccines and Immunotherapeutics* 13 (2): 351–58. https://doi.org/10.1080/21645515.2017.1264794.

Ten Berge, R. E. J., J. Zaagsma, and A. F. Roffel. 1996. "Muscarinic Inhibitory Autoreceptors in Different Generations of Human Airways." *American Journal of Respiratory and Critical Care Medicine* 154 (1): 43–49. https://doi.org/10.1164/ajrccm.154.1.8680697.

Ten Berge, R. E., M. Krikke, A. C. Teisman, A. F. Roffel, and J. Zaagsma. 1996. "Dysfunctional Muscarinic M2 Autoreceptors in Vagally Induced Bronchoconstriction of Conscious Guinea Pigs after the Early Allergic Reaction." *European Journal of Pharmacology* 318 (1): 131–39. https://doi.org/10.1016/s0014-2999(96)00820-5.

Thorneloe, K. S., M. Cheung, W. Bao, H. Alsaid, S. Lenhard, M. Y. Jian, M. Costell, et al. 2012. "An Orally Active TRPV4 Channel Blocker Prevents and Resolves Pulmonary Edema Induced by Heart Failure." *Science Translational Medicine* 4 (159): 159ra148. https://doi.org/10.1126/scitranslmed.3004276.

Thorneloe, K. S., A. C. Sulpizio, Z. Lin, D. J. Figueroa, A. K. Clouse, G. P. McCafferty, T. P. Chendrimada, et al. 2008. "GSK1016790A, a Novel and Potent Transient Receptor Potential Vanilloid 4 Channel Agonist Induces Urinary Bladder Contraction and Hyperactivity: Part I." *Journal of Pharmacology and Experimental Therapeutics* 326 (2):

432–42. https://doi.org/10.1124/jpet.108.139295.

Tränkner, D., N. Hahne, K. Sugino, M. A. Hoon, and C. Zuker. 2014. “Population of Sensory Neurons Essential for Asthmatic Hyperreactivity of Inflamed Airways.” *Proceedings of the National Academy of Sciences of the United States of America* 111 (31): 11515–20. https://doi.org/10.1073/pnas.1411032111.

Trischler, J., A. Lieb, M. Arnold, J. Schulze, M. Rosewich, R. Schubert, I. Bottoli, and S. Zielen. 2017. “Omalizumab Effectively Protects against Early and Late Allergic Responses in Asthma after 4 Weeks.” *Allergy* 72 (12): 1912–15. https://doi.org/10.1111/all.13217.

Undem, B. J., and M. J. Carr. 2002. “The Role of Nerves in Asthma.” *Current Allergy and Asthma Reports* 2 (2): 159–65. https://doi.org/10.1007/s11882-002-0011-4.

Undem, B. J., B. Chuaychoo, M. G. Lee, D. Weinreich, A. C. Myers, and M. Kollarik. 2004. “Subtypes of Vagal Afferent C-Fibres in Guinea-Pig Lungs.” *The Journal of Physiology* 556 (Pt 3): 905–17. https://doi.org/10.1113/jphysiol.2003.060079.

Undem, B. J., W. Hubbard, and D. Weinreich. 1993. “Immunologically Induced Neuromodulation of Guinea Pig Nodose Ganglion Neurons.” *Journal of the Autonomic Nervous System* 44 (1): 35–44. https://doi.org/10.1016/0165-1838(93)90376-6.

Undem, B. J., and C. Potenzieri. 2012. “Autonomic Neural Control of Intrathoracic Airways.” *Comprehensive Physiology* 2 (2): 1241–67. https://doi.org/10.1002/cphy.c110032.

Undem, B. J., and T. Taylor-Clark. 2014. “Mechanisms Underlying the Neuronal-Based Symptoms of Allergy.” *Journal of Allergy and Clinical Immunology* 133 (6): 1521–34. https://doi.org/10.1016/j.jaci.2013.11.027.

Underwood, S., M. Foster, D. Raeburn, S. Bottoms, and J. A. Karlsson. 1995. “Time-Course of Antigen-Induced Airway Inflammation in the Guinea-Pig and Its Relationship to Airway Hyperresponsiveness.” *European Respiratory Journal* 8 (12): 2104–13. https://doi.org/10.1183/109031936.95.08122104.

Underwood, S. L., E. Haddad, M. A. Birrell, K. McCluskie, M. Pecoraro, D. Dabrowski, S. E. Webber, M. L. Foster, and M. G. Belvisi. 2002. “Functional Characterization and Biomarker Identification in the Brown Norway Model of Allergic Airway Inflammation.” *British Journal of Pharmacology* 137 (2): 263–75. https://doi.org/10.1038/sj.bjp.0704865.

Vaickus, L. J., J. Bouchard, J. Kim, S. Natarajan, and D. G. Remick. 2010. “Assessing Pulmonary Pathology by Detailed Examination of Respiratory Function.” *American Journal of Pathology* 177 (4): 1861–69. https://doi.org/10.2353/ajpath.2010.100053.

Van Der Ploeg, E. K., A. Carreras Mascaro, D. Huylebroeck, R.W. Hendriks, and R. Stadhouders. 2020. “Group 2 Innate Lymphoid Cells in Human Respiratory Disorders.” *Journal of Innate Immunity* 12 (1): 47–62. https://doi.org/10.1159/000496212.

Van Der Wiel, E., N. H. T. Ten Hacken, D. S. Postma, and M. Van Den Berge. 2013. “Small-Airways Dysfunction Associates with Respiratory Symptoms and Clinical Features of Asthma: A Systematic Review.” *Journal of Allergy and Clinical Immunology* 131 (3): 646–57. https://doi.org/10.1016/j.jaci.2012.12.1567.

Van Hove, C. L., T. Maes, G. F. Joos, and K. G. Tournoy. 2007. “Prolonged Inhaled Allergen Exposure Can Induce Persistent Tolerance.” *American Journal of Respiratory Cell and Molecular Biology* 36 (5): 573–84. https://doi.org/10.1165/rcmb.2006-0385OC.

Verheijden, K. A. T., P. A. J. Henricks, F. A. Redegeld, J. Garssen, and G. Folkerts. 2014. “Measurement of Airway Function Using Invasive and Non-Invasive Methods in Mild and Severe Models for Allergic Airway Inflammation in Mice.” *Frontiers in Pharmacology* 5: 190. https://doi.org/10.3389/fphar.2014.00190.

Virchow, J. C., P. Kuna, P. Paggiaro, A. Papi, D. Singh, S. Corre, F. Zuccaro, et al. 2019. “Single Inhaler Extrafine Triple Therapy in Uncontrolled Asthma (TRIMARAN and TRIGGER): Two Double-Blind, Parallel-Group, Randomised, Controlled Phase 3 Trials.” *The Lancet* 394 (10210): 1737–49. https://doi.org/10.1016/S0140-6736(19)32215-9.

Vriens, J., H. Watanabe, A. Janssens, G. Droogmans, T. Voets, and B. Nilius. 2004. “Cell Swelling, Heat, and Chemical Agonists Use Distinct Pathways for the Activation of the Cation Channel TRPV4.” *Proceedings of the National Academy of Sciences of the United States of America* 101 (1): 396–401. https://doi.org/10.1073/pnas.0303329101.

Vrijlandt, E. J. L. E., G. El Azzi, M. Vandewalker, N. Rupp, T. Harper, L. R. Graham, S. J. Szefler, et al. 2018. “Safety and Efficacy of Tiotropium in Children Aged 1–5 Years with Persistent Asthmatic Symptoms: A Randomised, Double-Blind, Placebo-Controlled Trial.” *The Lancet Respiratory Medicine* 6 (2): 127–37. https://doi.org/10.1016/S2213-2600(18)30012-2.

Wang, L. L., H. P. Tang, G. C. Shi, H. Y. Wan, W. Tang, X. X. Hou, L. N. Pan, B. Y. Shi, and L. Q. Tao. 2013. “CD39/CD73 and the Imbalance of Th17 Cells and Regulatory T Cells in Allergic Asthma.” *Molecular Medicine Reports* 8 (5): 1432–38. https://doi.org/10.3892/mmr.2013.1692.

Wang, L. L., P. H. Tang, C. G. Shi, Y. H. Wan, W. Tang, X. X. Hou, N. L. Pan, Y. B. Shi, and Q. L. Tao. 2014. “Expression of CD39 MRNA Is Altered in the Peripheral Blood of Patients with Allergic Asthma.” *Biomedical Reports* 2 (1): 75–78. https://doi.org/10.3892/br.2013.196.

Wang, Wei, Yun Yan Li, Zhe Lv, Yan Chen, Yun Yan Li, Kewu Huang, Chris J. Corrigan, and Sun Ying. 2018. “Bronchial Allergen Challenge of Patients with Atopic Asthma Triggers an Alarmin (IL-33, TSLP, and IL-25) Response in the Airways Epithelium and Submucosa.” *The Journal of Immunology* 201 (8): 2221–31. https://doi.org/10.4049/jimmunol.1800709.

Watanabe, A., H. Mishima, P. M. Renzi, L. J. Xu, Q. Hamid, and J. G. Martin. 1995. “Transfer of Allergic Airway Responses with Antigen-Primed CD4+ but Not CD8+ T Cells in Brown Norway Rats.” *Journal of Clinical Investigation* 96 (3): 1303–10. https://doi.org/10.1172/JCI118165.

Watanabe, H., J. B. Davis, D. Smart, J. C. Jerman, G. D. Smith, P. Hayes, J. Vriens, et al. 2002. “Activation of TRPV4 Channels (HVRL-2/MTRP12) by Phorbol Derivatives.” *Journal of Biological Chemistry* 277 (16): 13569–77. https://doi.org/10.1074/jbc.M200062200.

Watanabe, H., J. Vriens, J. Prenen, G. Droogmans, T. Voets, and B. Nilius. 2003. “Anandamide and Arachidonic Acid Use Epoxyeicosatrienoic Acids to Activate TRPV4 Channels.” *Nature* 424 (6947): 434–38. https://doi.org/10.1038/nature01807.

Watanabe, H., J. Vriens, S. H. Suh, C. D. Benham, G. Droogmans, and B. Nilius. 2002. “Heat-Evoked Activation of TRPV4 Channels in a HEK293 Cell Expression System and in Native Mouse Aorta Endothelial Cells.” *Journal of Biological Chemistry* 277 (49): 47044–51. https://doi.org/10.1074/jbc.M208277200.

Watson, N., H. Magnussen, and K. F. Rabe. 1995. “Pharmacological Characterization of the Muscarinic Receptor Subtype Mediating Contraction of Human Peripheral Airways.” *The Journal of Pharmacology and Experimental Therapeutics* 274 (3): 1293–97. http://www.ncbi.nlm.nih.gov/pubmed/7562501.

Webb, D. C., A. N. J. McKenzie, A. M. L. Koskinen, M. Yang, J. Mattes, and P. S. Foster. 2000. “Integrated Signals Between IL-13, IL-4, and IL-5 Regulate Airways Hyperreactivity.” *The Journal of Immunology* 165 (1): 108–13.

https://doi.org/10.4049/jimmunol.165.1.108.

Weibel, E. R. 1963. *Morphometry of the Human Lung*. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-87553-3.

Weigand, L. A., A. P. Ford, and B. J. Undem. 2012. “A Role for ATP in Bronchoconstriction-Induced Activation of Guinea Pig Vagal Intrapulmonary C-Fibres.” *The Journal of Physiology* 590 (16): 4109–20. https://doi.org/10.1113/jphysiol.2012.233460.

Weigand, L. A., A. C. Myers, S. Meeker, and B. J. Undem. 2009. “Mast Cell-Cholinergic Nerve Interaction in Mouse Airways.” *The Journal of Physiology* 587 (13): 3355–62. https://doi.org/10.1113/jphysiol.2009.173054.

Weller, P. F., C. W. Lee, D. W. Foster, E. J. Corey, K. F. Austen, and R. A. Lewis. 1983. “Generation and Metabolism of 5-Lipoxygenase Pathway Leukotrienes by Human Eosinophils: Predominant Production of Leukotriene C4.” *Proceedings of the National Academy of Sciences of the United States of America* 80 (24 I): 7626–30. https://doi.org/10.1073/pnas.80.24.7626.

Wenzel, S., M. Castro, J. Corren, J. Maspero, L. Wang, B. Zhang, G. Pirozzi, et al. 2016. “Dupilumab Efficacy and Safety in Adults with Uncontrolled Persistent Asthma despite Use of Medium-to-High-Dose Inhaled Corticosteroids plus a Long-Acting B2 Agonist: A Randomised Double-Blind Placebo-Controlled Pivotal Phase 2b Dose-Ranging Trial.” *The Lancet* 388 (10039): 31–44. https://doi.org/10.1016/S0140-6736(16)30307-5.

Wenzel, S. E., A. A. Fowler, and L. B. Schwartz. 1988. “Activation of Pulmonary Mast Cells by Bronchoalveolar Allergen Challenge. In Vivo Release of Histamine and Tryptase in Atopic Subjects with and without Asthma.” *The American Review of Respiratory Disease* 137 (5): 1002–8. https://doi.org/10.1164/ajrccm/137.5.1002.

Wenzel, S., D. Wilbraham, R. Fuller, E. B. Getz, and M. Longphre. 2007. “Effect of an Interleukin-4 Variant on Late Phase Asthmatic Response to Allergen Challenge in Asthmatic Patients: Results of Two Phase 2a Studies.” *Lancet* 370 (9596): 1422–31. https://doi.org/10.1016/S0140-6736(07)61600-6.

West, P. W., B. J. Canning, E. Merlo-Pich, A. A. Woodcock, and J. A. Smith. 2015. “Morphologic Characterization of Nerves in Whole-Mount Airway Biopsies.” *American Journal of Respiratory and Critical Care Medicine* 192 (1): 30–39. https://doi.org/10.1164/rccm.201412-2293OC.

Westby, M. J., M. K. Benson, and P. G. Gibson. 2004. “Anticholinergic Agents for Chronic Asthma in Adults.” *Cochrane Database of Systematic Reviews*. https://doi.org/10.1002/14651858.CD003269.pub2.

White, J. P. M., M. Cibelli, L. Urban, B. Nilius, J. G. McGeown, and I. Nagy. 2016. “TRPV4: Molecular Conductor of a Diverse Orchestra.” *Physiological Reviews* 96 (3): 911–73. https://doi.org/10.1152/physrev.00016.2015.

Widdicombe, J. G. 1966. “Action Potentials in Parasympathetic and Sympathetic Efferent Fibres to the Trachea and Lungs of Dogs and Cats.” *The Journal of Physiology* 186 (1): 56–88. https://doi.org/10.1113/jphysiol.1966.sp008020.

———. 1982. “Pulmonary and Respiratory Tract Receptors.” *Journal of Experimental Biology* 100: 41–57.

———. 2003. “Functional Morphology and Physiology of Pulmonary Rapidly Adapting Receptors (RARs).” *The Anatomical Record* 270A (1): 2–10. https://doi.org/10.1002/ar.a.10003.

Willette, R. N., W. Bao, S. Nerurkar, T. L. Yue, C. P. Doe, G. Stankus, G. H. Turner, et al.

2008. “Systemic Activation of the Transient Receptor Potential Vanilloid Subtype 4 Channel Causes Endothelial Failure and Circulatory Collapse: Part 2.” *The Journal of Pharmacology and Experimental Therapeutics* 326 (2). https://doi.org/10.1124/jpet.107.134551.

Wine, J. J. 2007. “Parasympathetic Control of Airway Submucosal Glands: Central Reflexes and the Airway Intrinsic Nervous System.” *Autonomic Neuroscience: Basic and Clinical* 133 (1): 35–54. https://doi.org/10.1016/j.autneu.2007.01.008.

Winkler, Carla, Thomas Hochdörfer, Elisabeth Israelsson, Annemarie Hasselberg, Anders Cavallin, Kristofer Thörn, Daniel Muthas, et al. 2019. “Activation of Group 2 Innate Lymphoid Cells after Allergen Challenge in Asthmatic Patients.” *Journal of Allergy and Clinical Immunology* 144 (1): 61-69.e7. https://doi.org/10.1016/j.jaci.2019.01.027.

Woehrle, T., L. Yip, A. Elkhal, Y. Sumi, Y. Chen, Y. Yao, P. A. Insel, and W. G. Junger. 2010. “Pannexin-1 Hemichannel-Mediated ATP Release Together with P2X1 and P2X4 Receptors Regulate T-Cell Activation at the Immune Synapse.” *Blood* 116 (18): 3475–84. https://doi.org/10.1182/blood-2010-04-277707.

Wohlsen, A., C. Martin, E. Vollmer, D. Branscheid, H. Magnussen, W. M. Becker, U. Lepp, and S. Uhlig. 2003. “The Early Allergic Response in Small Airways of Human Precision-Cut Lung Slices.” *European Respiratory Journal* 21 (6): 1024–32. https://doi.org/10.1183/09031936.03.00027502.

Wortley, M. A., E. Dubuis, S. J. Bonvini, S. Wong, F. Shala, S. A. Maher, J. J. Adcock, J. A. Smith, M. A. Birrell, and M. G. Belvisi. 2016. “Making Sense of Sensory Nerves: An In Vitro Characterisation of Gene Expression Profiles of Airway-Innervating Guinea-Pig Airway Neurons Using Single-Cell Analysis.” *Am J Respir Crit Care Med* 193: A6002. https://www.atsjournals.org/doi/abs/10.1164/ajrccm-conference.2016.193.1_MeetingAbstracts.A6002.

Wright, A., M. Mahaut-Smith, F. Symon, N. Sylvius, S. Ran, M. Bafadhel, M. Muessel, P. Bradding, A. Wardlaw, and C. Vial. 2016. “Impaired P2X1 Receptor–Mediated Adhesion in Eosinophils from Asthmatic Patients.” *The Journal of Immunology* 196 (12): 4877–84. https://doi.org/10.4049/jimmunol.1501585.

Yamamoto, Y., M. Hayashi, Y. Atoji, and Y. Suzuki. 1994. “Vagal Afferent Nerve Endings in the Trachealis Muscle of the Dog.” *Archives of Histology and Cytology* 57 (5): 473–80. https://doi.org/10.1679/aohc.57.473.

Yancey, S. W., H. G. Ortega, O. N. Keene, B. Mayer, N. B. Gunsoy, C. E. Brightling, E. R. Bleecker, P. Haldar, and I. D. Pavord. 2017. “Meta-Analysis of Asthma-Related Hospitalization in Mepolizumab Studies of Severe Eosinophilic Asthma.” *Journal of Allergy and Clinical Immunology* 139 (4): 1167-1175.e2. https://doi.org/10.1016/j.jaci.2016.08.008.

Yau, M. K., L. Liu, and D. P. Fairlie. 2013. “Toward Drugs for Protease-Activated Receptor 2 (PAR2).” *Journal of Medicinal Chemistry* 56 (19): 7477–97. https://doi.org/10.1021/jm400638v.

Yau, Mei-Kwan, Junxian Lim, Ligong Liu, and David P. Fairlie. 2016. “Protease Activated Receptor 2 (PAR2) Modulators: A Patent Review (2010–2015).” *Expert Opinion on Therapeutic Patents* 26 (4): 471–83. https://doi.org/10.1517/13543776.2016.1154540.

Yeh, H. C., G. M. Schum, and M. T. Duggan. 1979. “Anatomic Models of the Tracheobronchial and Pulmonary Regions of the Rat.” *The Anatomical Record* 195 (3): 483–92. https://doi.org/10.1002/ar.1091950308.

Yoshida, M., R. M. Watson, T. Rerecich, and P. M. O’Byrne. 2005. “Different Profiles of T-Cell

IFN-Gamma and IL-12 in Allergen-Induced Early and Dual Responders with Asthma." *The Journal of Allergy and Clinical Immunology* 115 (5): 1004–9. https://doi.org/10.1016/j.jaci.2005.02.003.

Yu, D. Y., S. P. Galant, and W. M. Gold. 1972. "Inhibition of Antigen-Induced Bronchoconstriction by Atropine in Asthmatic Patients." *J. Appl. Physiol.* 32 (6): 823–28. https://doi.org/10.1152/jappl.1972.32.6.823.

Yu, J, H D Schultz, J Goodman, J C Coleridge, H M Coleridge, and B Davis. 1989. "Pulmonary Rapidly Adapting Receptors Reflexly Increase Airway Secretion in Dogs." *Journal of Applied Physiology (Bethesda, Md.: 1985)* 67 (2): 682–87. https://doi.org/10.1152/jappl.1989.67.2.682.

Zech, A., B. Wiesler, C. K. Ayata, T. Schlaich, T. Dürk, M. Hoßfeld, N. Ehrat, S. Cicko, and M. Idzko. 2016. "P2rx4 Deficiency in Mice Alleviates Allergen-Induced Airway Inflammation." *Oncotarget* 7 (49): 80288–97. https://doi.org/10.18632/oncotarget.13375.

Zhang, Guangfan, Ruei Lung Lin, Michelle E. Wiggers, and Lu Yuan Lee. 2008. "Sensitizing Effects of Chronic Exposure and Acute Inhalation of Ovalbumin Aerosol on Pulmonary C Fibers in Rats." *Journal of Applied Physiology* 105 (1): 128–38. https://doi.org/10.1152/japplphysiol.01367.2007.

Zhang, J., Y. Wei, S. Bai, S. Ding, H. Gao, S. Yin, S. Chen, et al. 2019. "TRPV4 Complexes With the Na+/Ca2+ Exchanger and IP3 Receptor 1 to Regulate Local Intracellular Calcium and Tracheal Tension in Mice." *Frontiers in Physiology* 10: 1471. https://doi.org/10.3389/fphys.2019.01471.

Zhang, L., S. Jones, K. Brody, M. Costa, and S. J. H. Brookes. 2004. "Thermosensitive Transient Receptor Potential Channels in Vagal Afferent Neurons of the Mouse." *American Journal of Physiology - Gastrointestinal and Liver Physiology* 286 (6 49-6). https://doi.org/10.1152/ajpgi.00441.2003.

Zhao, P., T. Lieu, N. Barlow, M. Metcalf, N. A. Veldhuis, D. D. Jensen, M. Kocan, et al. 2014. "Cathepsin S Causes Inflammatory Pain via Biased Agonism of PAR2 and TRPV4." *Journal of Biological Chemistry* 289 (39): 27215–34. https://doi.org/10.1074/jbc.M114.599712.

Zhao, P., T. M. Lieu, N. Barlow, S. Sostegni, S. Haerteis, C. Korbmacher, W. Liedtke, N. N. Jimenez-Vargas, S. J. Vanner, and N. W. Bunnett. 2015. "Neutrophil Elastase Activates Protease-Activated Receptor-2 (PAR2) and Transient Receptor Potential Vanilloid 4 (TRPV4) to Cause Inflammation and Pain." *Journal of Biological Chemistry* 290 (22): 13875–87. https://doi.org/10.1074/jbc.M115.642736.

Zhong, Y., P. M. Dunn, M. Bardini, A. P. Ford, D. A. Cockayne, and G. Burnstock. 2001. "Changes in P2X Receptor Responses of Sensory Neurons from P2X3-Deficient Mice." *The European Journal of Neuroscience* 14 (11): 1784–92. https://doi.org/10.1046/j.0953-816x.2001.01805.x.

Zosky, G. R., and P. D. Sly. 2007. "Animal Models of Asthma." *Clinical & Experimental Allergy* 37 (7): 973–88. https://doi.org/10.1111/j.1365-2222.2007.02740.x.

Appendix

The table below outlines reagents used in this thesis.

Reagent	Source	Vehicle (where applicable)
αβ-MeATP	Sigma-Aldrich, USA	0.1% H_2O in KH *in vitro*
2f-LIGRLO-NH2	Tocris, UK	0.1% DMSO in KH/ECS *in vitro* 0.1% DMSO in saline *in vivo*
5-BDBD	Tocris, UK	0.1% DMSO in ECS *in vitro*
AC55541	Tocris, UK	
ACh	Sigma-Aldrich, USA	KH/ECS *in vitro* Saline *in vivo*
AF-353	Sigma-Aldrich, USA	0.1% DMSO in KH/ECS *in vitro* 10% PEG400 in saline *in vivo*
Amphotericin B	Sigma-Aldrich, USA	
ATP	Sigma-Aldrich, USA	1% H_2O in ECS *in vitro*
BSA	Sigma-Aldrich, USA	
$CaCl_2$	VWR, USA	
Capsaicin	Sigma-Aldrich, USA	0.1% DMSO in KH *in vitro*
Carbachol	Sigma-Aldrich, USA	KH/ECS *in vitro*
HP-β-cyclodextrin	Sigma-Aldrich, USA	
Citric acid	Sigma-Aldrich, USA	Saline
DMEM high glucose	ThermoFisher Scientific, USA	
DMSO	Sigma-Aldrich, USA	
Ethanol	VWR, USA	
FBS	ThermoFisher Scientific, USA	
Glucose	Sigma-Aldrich, USA	
GSK1016790A	Sigma-Aldrich, USA	0.1% DMSO in KH/ECS *in vitro* 1% EtOH in saline *in vivo*
GSK2193874	Sigma-Aldrich, USA	0.1% DMSO in KH/ECS *in vitro* 6% HP-β-cyclodextrin in saline *in vivo*
HBSS	Sigma-Aldrich, USA	
HEPES	Sigma-Aldrich, USA	
Imject Alum™	ThermoFisher Scientific, USA	
Indomethacin	Sigma-Aldrich, USA	0.1 mM Na_2CO_3 in KH *in vitro*
Isoflurane	Abbott laboratories, USA	
KCl	VWR, USA	
L-Glutamine	Sigma-Aldrich, USA	
MEM Non-Essential Amino Acid Solution	Sigma-Aldrich, USA	
Mepyramine maleate	Sigma-Aldrich, USA	Saline
Methylcellulose	Sigma-Aldrich, USA	
Methysergide maleate	Sigma-Aldrich, USA	Saline
$MgSO_4$	VWR, USA	
Montelukast (sodium salt)	Cayman Chemical, USA	Methylcellulose 0.5% Tween80 0.2% in saline *in vivo*
NaCl	VWR, USA	
NaH_2PO_4	VWR, USA	

$NaHCO_3$	Sigma-Aldrich, USA	
Nuclease free water	Promega, USA	
Ovalbumin from chicken egg white (OVA)	Sigma-Aldrich, USA	1:1 Alum™:saline
P2pal-18S	AstraZeneca, Sweden	0.1% DMSO in KH *in vitro* 5% DMSO in saline *in vivo*
Pancuronium bromide	Sigma-Aldrich, USA	
PEG400	Sigma-Aldrich, USA	
Penicillin	Sigma-Aldrich, USA	
Probenecid water soluble	ThermoFisher Scientific, USA	0.1% DMSO in KH/ECS *in vitro*
RPMI 1640 Glutamax	ThermoFisher Scientific, USA	
Saline (0.9% NaCl)	Fresenius Kabi, Germany	
Scrambled P2pal-18S	AstraZeneca, Sweden	0.1% DMSO in KH *in vitro* 5% DMSO in saline *in vivo*
SLIGKV-NH2	Tocris, UK	
Sodium pentobarbitone	Marial Animal Health, France	
Streptomycin	Sigma-Aldrich, USA	
Tiotropium bromide	Sigma-Aldrich, USA	0.5% EtOH in saline *in vivo*
Trypsin	Sigma-Aldrich, USA	0.1% H_2O in KH *in vitro*
Tween80	Sigma-Aldrich, USA	
Urethane	Sigma-Aldrich, USA	