

Investigation Into the Mechanism of Action of Disodium Cromoglycate in Pre-clinical Models of Asthma

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A thesis submitted in fulfilment of the requirements for the degree
of Doctor of Philosophy, Imperial College London

2020

Abstract

Asthma is a disease with increasing global prevalence and patients typically suffer from symptoms such as wheezing, breathlessness, chest tightness and cough. Although in most cases asthma can be well controlled with current treatments, there remains an unmet need as patients do not always comply with therapies, and some remain unresponsive to treatment. In allergic asthma, exposure to allergen can lead to episodes of bronchoconstriction known as the late asthmatic response (LAR), a characteristic feature of asthma which has been shown to involve the activation of airway sensory nerves in a preclinical model. In asthmatics, Disodium Cromoglycate (DSCG) is known to modulate the LAR but its use is limited because of its short duration of action seen evidenced by its pharmacokinetic profile. Although a safe and effective drug, the mechanism of action of DSCG remains unclear. The aim of this thesis is to investigate the mechanism with the possibility to develop a novel therapy with an improved PK profile and thus be a more useful treatment.

Using a pre-clinical Brown Norway (BN) rat model of the LAR, it was established that both DSCG and a TRPA1 inhibitor can attenuate the LAR, suggesting an association between DSCG and TRPA1 on airway sensory nerves. This was confirmed by using an *in vitro* model of vagal sensory nerve activation, an *in vitro* model of airway specific neuron activation, *in vivo* sensory nerve recordings and an *in vivo* cough model, which all showed that DSCG can attenuate TRPA1 induced activation of airway sensory nerves.

While investigating the mechanism by which DSCG inhibits airway sensory nerves, two distinct mechanisms were uncovered which depended on how the channel was opened. The first was that DSCG inhibited TRPA1 activity on airway sensory nerves through agonising GPR35 receptors. Selective GPR35 agonists demonstrated a similar inhibitory effect as DSCG, and this was reversed with GPR35 antagonists. Investigations showed that GPR35 was only responsible for some of the activity of DSCG on sensory nerves (i.e. with acrolein as the trigger); some DSCG modulated TRPA1 triggers were independent of GPR35 (i.e. PGE₂). The second mechanism appeared to be via the blockade of TRPV2 channel/signalling and subsequent reduction of NADPH oxidase activity.

The work within my thesis has therefore uncovered the important role of GPR35, TRPV2 and NADPH oxidase activity in the mechanism of action of DSCG by using pre-clinical models. These findings help to explain the beneficial effects observed in the clinic and also highlight novel targets for drug discovery.

Acknowledgements

I would like to thank my supervisor Professor Maria Belvisi and Dr Mark Birrell for giving me the opportunity to work in the Respiratory Pharmacology group and their continual guidance and inspiration. I have learnt so much both scientifically and culturally in the journey of PhD. I would also like to thank Professor Joshua Edel and Professor Keith Willison for their kind support and encouragement.

I am grateful for meeting a group of amazing people who made this PhD fruitful and enjoyable. Sara, my 'bench' supervisor, gave me the most help in these years. Eric, the BFG, spread interesting science and tech knowledge. John, best artist among scientists (or the other way round), inspired me with so many stories in science and art. Bilel, best technician in the world, taught me how to do experiments and kindly shared his cat Oreo for lovely company. Pauline, my lab buddy, provided me emotional support and delicious French food. Mike, I like your unique sense of humour and you can always provide new angles to interpret questions. This page is short, and I would like to send my thank you to Jono, Nadja, Thomais, James B and P, Mark F, Dimitris, etc.

I would also like to thank the 'Klug Krazy Kensington Krew' and 'Emperor's Gate Gang' for providing lovely company and support in life. I would like to thank London for being an amazing city and making my years colourful.

Thank you to my parents (although they probably would not read my thesis) for their unconditional support and love.

Statement of Originality

The work contained in this thesis is my own work, except where otherwise indicated and referenced appropriately. The single fibre experiments carried out in Chapters 3, 4 and 5 were performed by Dr John Adcock, with my assistance.

Declaration

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List of Abbreviations

ACh	Acetylcholine
AHR	Airway Hyperresponsiveness
APC	Antigen Presenting Cell
AUC	Area Under Curve
BAL	Bronchoalveolar lavage (fluid)
B2 receptor	Bradykinin receptor B2
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
Dil	DilC ₁₈ (3) (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate
DMSO	Dimethyl Sulfoxide
DSCG	Disodium Cromoglycate
EAR	Early Asthmatic Response
EP ₃ receptor	Prostaglandin EP ₃ receptor
FcεR	Fc-epsilon receptor
FWBP	Flow Whole Body Plethysmography
GI	Gastrointestinal
GPR35	G-protein Coupled Receptor 35
HDM	House Dust Mite
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
ICS	Inhaled Corticosteroid
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
K50	50mM potassium chloride
LABA	Long Acting β ₂ -adrenoceptor Agonist
LAMA	Long Acting Muscarinic Receptor Antagonist
LAR	Late Asthmatic Response
M (1, 2, 3)	Muscarinic receptor (subtypes 1, 2, 3)
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
mOsm	milli-osmole
NADP	Nicotinamide adenine dinucleotide phosphate
NKA	Neurokinin A
NTS	Nucleus Tractus Solitarius
OVA	Ovalbumin
OTC	Over The Counter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Penh	Enhanced pause
PGE ₂	Prostaglandin E ₂
PGP9.5	Protein Gene Product 9.5
RARs	Rapidly Adapting Stretch Receptors
ROS	Reactive Oxygen Species

SABA	Short Acting β 2-adrenoceptor Agonist
SARs	Slowly Adapting Stretch Receptors
s.c.	Subcutaneous
S.E.M	Standard error of the mean
SP	Substance P
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
TRPM8	Transient receptor potential cation channel, subfamily M, member 8
TRPV1	Transient receptor potential cation channel, subfamily V, member 1
TRPV4	Transient receptor potential cation channel, subfamily V, member 4
w/v	Weight/volume
v/v	Volume/volume
5-HT	5-hydroxytryptamine

Chapter 1 Introduction

1.1 Asthma

1.1.1 Epidemiology

Asthma is a chronic inflammatory airway disease, defined by symptoms such as wheezing, coughing, chest tightness and difficulty breathing¹. Presently, asthma is one of the most prevalent chronic airway diseases in the world, with approximately 300 million individuals affected², and 346,000 yearly deaths³. The prevalence of asthma is still increasing year-on-year, and the cost of healthcare related to asthma diagnosis and treatment has become a global health concern³. A study by Asthma UK Centre has discovered that in the UK, one of the countries where allergy and asthma are the most prevalent, asthma costs the health service more than £1.1 billion each year⁴. Given that asthma was highlighted as a global burden, it is therefore of utmost importance to develop novel treatments.

Clinically, asthma is characterised as a heterogeneous syndrome which ranges from mild disease with hardly observable symptoms to very serious disease with persistent symptoms⁵. In terms of triggers, asthma patients can be responsive to a broad range of them, such as allergens, exercise and cold air. The patients can also have different sensitivities to treatment. Asthma can be roughly divided into atopic and non-atopic. My thesis focuses on atopic (allergic) asthma. It is the most common phenotype (45%-88%) in all patients relating to allergy and allergy elevated levels of allergen specific IgE⁶. Therefore, in the development of asthma, allergic sensitisation is identified as a key determinant. Pollen, animal dander and house dust mite were identified as the most common etiological allergens to cause atopic asthma⁷. In contrast, non-atopic (non-allergic) asthma is unrelated to allergy triggers and is also less common than atopic asthma. The causes are not well understood, but it often develops later in life, and can be more severe⁸.

1.1.2 Pathophysiology

In atopic asthma, the Th2 phenotype is a key immunological feature and factor in the pathogenesis of the disease where it dominates the allergic immune response and is most likely to be responsible for ongoing chronic inflammation. The immune response is initiated with a phase of sensitisation, followed by a challenge or allergic response phase. In association with the airway epithelium and underlying mucosa is a specialised population of antigen-presenting cells (APCs)⁹. In allergen sensitisation, APCs express receptors of the innate immune system and process allergens into small peptides and then present them through the major histocompatibility complexes, MHC class I and MHC class II for recognition by naive T cells or B cells. A complex formed between the MHC-II/allergen molecule and T cells expresses the specific T cell receptor (TCR). Accordingly, the antigen must be presented to the relevant T cell that expresses the right TCR. However, each TCR can in fact recognise

a number of different antigenic peptides¹⁰. This complex formation and additional co-stimulatory responses subsequently leads to the proliferation and differentiation of T cells, depending on the cytokine environment where antigen presentation occurs. The differentiation predominantly results in Th1 or Th2 effector cells. As aforementioned, T cells changing into Th2 cells is an important immunological feature of asthma. Cytokines such as interleukin 4 (IL-4) released from APCs during presentation promote Th2 polarisation while cytokines such as interferon- γ (IFN- γ) promote Th1 polarisation. It is the Th2 cells that then release IL-4 and IL-13 which in turn stimulates B cells to produce and release allergen specific IgE. These IgE molecules bind to high affinity Fc ϵ receptors (Fc ϵ R1) expressed on mast cells and basophils for future allergen exposure^{11,12}.

Further allergen exposure following sensitisation results in an allergic response. The allergen stimulates sensitised mast cells by inducing the cross linking of the IgE-Fc ϵ R1 complexes which lead to the release of mediators such as histamine, prostaglandins, 5-hydroxytryptamine (5-HT), and leukotrienes. These mediators can cause physiological responses that typically contribute to rapid bronchoconstriction, oedema and mucus secretion, which all contribute to airflow obstruction. This immediate bronchoconstriction, which typically happens within 1 hour after allergen exposure, is called the Early Asthmatic Response (EAR)⁷. The Late Asthmatic Response (LAR) is a recurrence of bronchoconstriction event that takes place approximately 3 – 8 hours following allergen exposure, lasting for 12 to 24 hours and sometimes longer. The LAR is characterised by an influx of inflammatory cells such as eosinophils, mast cells, neutrophils and T cells¹³. About 60% of asthmatics who experience the EAR will experience a LAR. In comparison to the aetiology of EAR, the LAR aetiology is less well understood^{14,15}. It is also of note that the LAR could be a more relevant marker in asthma than the EAR as firstly bronchoconstriction tends to be more pronounced and long-lived, and secondly the LAR is often used as an endpoint in asthma clinical studies^{16,17,18}.

Although it is not definitively confirmed, it is likely that the LAR is driven by inflammation at least in part^{19,20}. This comes from the observation that the LAR is associated with pulmonary inflammation^{21,22,23}, and the fact that Inhaled corticosteroids (ICS) treatment decreases the LAR^{24,25,26}. However our previous research has reported that the LAR is not driven by the mediators (e.g. 5-HT or histamine) which drive the EAR, but does involve the activation of sensory nerves and a following cholinergic bronchoconstriction²⁷. More evidence relating to the role of sensory nerves in this event will be discussed in section 1.2.

1.1.3 Current treatment

Current asthma treatment falls into two main categories: Firstly, bronchodilators are used to relieve symptoms and prevent airflow obstruction. Short and long acting β_2 -adrenoreceptor agonists such as

salbutamol or salmeterol/formoterol are typical bronchodilators that could be prescribed. Secondly, corticosteroids are used to control chronic inflammation in asthma. Inhaled corticosteroids (ICS) such as budesonide or fluticasone are most commonly used. Short acting β_2 -adrenoreceptor agonists (SABAs) attenuate allergen-induced EAR but not the LAR or allergen-induced increased reactivity to histamine²⁴. Long acting β_2 -adrenoreceptor agonists (LABAs) inhibit allergen-induced LAR and EAR²⁸. Corticosteroids inhibited the allergen-induced LAR to histamine but not the EAR¹⁴. Although the therapies are well developed and used, both LABA and steroids are associated with side-effects. There is evidence which shows that LABAs taken alone can increase the risk of mortality in asthma²⁹. The side effects of steroids can be dysphonia, thrush etc. Combining the LABA with ICS can reduce the risk of mortality³⁰. In addition, combining the LABA with ICS has been shown to be effective against clinical outcomes and asthma control and has beneficial effects over ICS only^{31,32}. It is known that β_2 -adrenoreceptor agonists and corticosteroids may promote each other's effect: steroids may prevent desensitisation of β_2 -adrenoreceptors³³ and β_2 -adrenoreceptor agonists may increase the efficacy of glucocorticoids and have some anti-inflammatory efficacy themselves providing further rationale for the combined treatment^{34,35}. However, another study contradicted this as it was suggested that β_2 -adrenoreceptor agonists may act to attenuate glucocorticoid efficacy³⁶.

Clinical treatment of asthma follows a stepwise manner (table 1.1): Patients with mild intermittent symptoms are recommended to use low dose ICS-formoterol as needed to relieve the symptoms. Patients with mild persistent symptoms are treated with daily low dose ICS or low dose ICS-formoterol as needed. More severe cases receive a combination of low dose ICS and long acting β_2 -adrenoreceptor agonists (LABA) such as salmeterol or formoterol. Severe persistent patients are treated with medium dose of ICS with LABA. If symptoms are still not under control, higher dose of ICS with LABA are used and add-on therapies are used into treatment such as tiotropium, anti-IgE, anti-IL5/5R, anti-IL4R³⁷. Although asthma can be controlled under these treatments, adherence to them is poor and inhaler use is often inadequate³⁸. Around 60% of asthma patients are difficult to manage, which has a big impact on their health-related quality of life³⁹. Therefore, these patients have an urgent need for new therapeutics.

	Severity of Asthma				
	Mild Intermittent	Mild Persistent	Moderate Persistent	Severe Persistent	Very Severe Persistent
Reliever	As-need low dose ICS-formoterol		As-need low dose ICS-formoterol for patients prescribed maintenance and reliever therapy		
Controller	As-need low dose ICS-formoterol	Daily low dose inhaled corticosteroid or as-need low dose ICS-formoterol	Low dose ICS	Medium dose ICS	High dose ICS
			LABA (combination inhaler)		
				LABA	LABA
					Add-on therapy: e.g. tiotropium, anti-IgE, anti-IL5/5R, anti-IL4R

Table 1.1 Patients' treatment according to the severity of their asthma.

LABA, long acting β_2 -adrenoreceptor agonists; ICS, inhaled corticosteroids.

1.2 Cough

Cough is a key symptom of asthma, exhibited by almost all asthmatic patients⁴⁰. It is key to the diagnosis and management of asthma². Cough is a protective reflex. In its normal state, this reflex serves a defensive purpose with a function to remove exogenous material and secretions from the airways. But under some disease conditions, inappropriate modulation of the cough reflex pathway can result in an augmented cough response. A cough reflex physiologically involves three phases. The first phase is a rapid deep inhalation via a widely open glottis. The second phase is the closure of the glottis, followed by an increase of intrathoracic pressure. The final phase is a rapid expiration with irritant materials removed from airways and lungs. Clinically, the aetiology and treatment of cough can be classified into acute (lasting less than 8 weeks) and chronic (over 8 weeks). Chronic cough is a symptom that can be present in conjunction with a number of respiratory diseases including not only asthma, but also chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and lung cancer amongst others⁴¹. There can also be unexplained or idiopathic coughs, of which the incidence may be 18% or higher of all patients at specialist cough clinics⁴².

1.2.1 Incidence and burden of cough in respiratory disease

Cough is the most common reason for patients to seek medical consultation⁴³. Several epidemiological researches have reported that chronic cough can occur up to 40% of the population at any one

time^{44,45}. Chronic cough is a common symptom of inflammatory diseases such as asthma where cough is a key factor leading to diagnosis⁴⁶. In addition, some asthma patients have cough variant asthma. Among these patients, cough is the main symptom, and other symptoms may not exist⁴⁷. Patients may be affected for months or years, which can lower quality of life if the symptom is not under control⁴⁸.

Apart from asthma, cough is also a burden in other respiratory diseases such as COPD and IPF. About 70% of COPD patients experienced cough as a symptom and 40% of them suffer from cough every day⁴⁹. The prevalence of cough in IPF has been reported to be 80%, and it is an independent predictor of disease severity, time to death, or need for lung transplantation⁵⁰.

Chronic cough can cause sleep disturbance, cough syncope, nausea and urinary incontinence in the long term^{48,51}. In addition, chronic cough can also severely affect social activities with friends and family, which can cause depression^{52,53}.

1.2.2 Current treatment

Normally cough associated with respiratory disease such as asthma can be treated by first treating the disease⁵⁴. However this is not always the case, and some cases remain treatment refractory. In some asthmatics with chronic cough, taking gold standard asthma therapies such as LABA and ICS does not always help with their cough⁵⁴. In addition, cough in diseases such as COPD and IPF are progressive and not stopped by therapy⁵⁰, and idiopathic cough has no known underlying respiratory disease that can be treated⁴². Therefore, there is a current line of thinking that chronic cough should be treated separately with specific anti-tussives.

Over the counter medications (OTCs) has been widely used as antitussive medications. The global market for over-the-counter (OTC) medicines in the category cough/cold is enormous, amounting to 32.81 billion dollar in 2019⁵⁵. However there is little evidence for the efficacy of OTC cough medicines. A recent systematic review of more than 2,100 participants failed to confirm the efficacy of OTC medications for cough⁵⁶, and a study showed that cough syrup has no pharmacological effect but instead works by acting as a lubricant for the throat.⁵⁷ At present, the only antitussive drugs that can show efficacy are drugs that act through the central nervous system, such as opioids. A clinical study for chronic cough patients showed that morphine sulphate is effective in patients who were non-responsive to other treatments⁵⁸. Another opioid antitussives, codeine, is currently the reference cough medicine, although studies have shown that codeine is not more effective than placebo in treating cough in patients with COPD⁵⁹. In addition, opioids have a series of potentially dangerous side effects, such as respiratory depression, sedation, and gastrointestinal problems⁶⁰. Therefore, the treatment of cough is still an unmet medical need, and new treatment methods will have great value for patients with chronic cough.

1.3 Airway reflexes and innervation of the airways and lungs

Key asthma symptoms, such as bronchospasm and cough, are respiratory reflexes initiated following activation of airway sensory nerves. The airways are innervated by several types of nerve fibres⁶¹, which sense the lung environment and relay pulmonary sensory information to the central nervous system (CNS) to initiate protective reflex events if activated⁵⁴. In chronic airway diseases such as asthma, these reflexes are thought to be upregulated leading to an increase in symptoms, and a number of studies have also suggested that there are interactions between nerves and the inflammation associated with asthma^{62–64} and so investigating airway innervation is key to understand the mechanisms driving the LAR and cough.

The vagus nerves supply all parasympathetic nerve fibres and most of the sensory nerve fibres to the airways⁶⁵, with a small number of fibres (approximately 1%) originating from the dorsal root ganglia and run with spinal sympathetic nerves. Vagal sensory nerve fibres' cell bodies are in the jugular and nodose ganglia, which have different embryological origins, where the jugular is neural crest derived and the nodose is derived from the epibranchial placode and as a result they house different populations of nerve fibres⁵⁴. The sensory nerves run centrally to the medulla and peripherally to the airways. Symptoms of allergic diseases, such as sneezing, coughing, mucus hypersecretion and bronchoconstriction can be due to improper activation of these nerves^{66,67}. The airway nerve abnormal functioning in inflammatory disease is thought to involve the release of mediators that sensitise or activate the nerve fibres, leading to increased electrical activity and release of various neurotransmitters from the nerve endings⁶⁸. In the following paragraph, different types of nerve fibres will be introduced as well as the pharmacological manipulation of airway neurotransmission.

1.3.1 Afferent pathways (airway sensory nerves)

Afferent airway sensory nerves are important in the control of airway tone. Allergen challenge can be associated with the activation of afferent nerve terminals leading to the generation of action potential trains signalling to the brain⁶⁹. Initially, certain allergens can lead to a localised membrane depolarisation at the nerve terminus, known as a generator potential. The magnitude of generator potential is positively correlated with factors such as the number of receptors and potency of receptor activation. If the generator potential reaches a certain threshold, an action potential is initiated where a rapid change in membrane potential is transmitted without decrement along the length of the cell. The threshold of an action potential is associated with the gating potential of the type of voltage gated sodium channels (VGSCs). The normal resting potential of a neuron is -70mV. Once VGSCs opens, it causes an influx of Na⁺. Then the potential reaches a threshold value (between -60 and -50mV), the

membrane becomes less negative as it depolarises, which generates an action potential. The VGSCs then become inactive to stop further depolarisation of neuron membranes. Afterwards an efflux of K^+ ions occurs due to the opening of voltage gated potassium channels (VGKCs), which causes repolarisation of the membrane. This wave of action potential can be propagated along the axon with a fixed size and shape. The axon transmits action potentials from their airway endings to their central endings in the brainstem, where an effector response can occur via motor neurons to cause cough or via parasympathetic efferent neurons to cause bronchospasm^{69,70}.

Afferent fibres are classified based on their functional properties into the following subtypes: slowly adapting stretch receptors (SARs), rapidly adapting stretch receptors (RARs), and C-fibre receptors⁶⁵.

SARs are myelinated fibres derived from the nodose ganglia. They have high conduction velocities, and are primarily involved in the control of breathing pattern⁷¹. SARs are responsible for the Hering–Breuer inflation reflex, a reflex triggered to prevent the over-inflation of the lung during large inspirations, by terminating inspiration and allowing expiration to occur. During excessive stretching of the lung, the pulmonary stretch receptors on the wall of bronchi and bronchioles that respond to excessive stretching of the lung are activated. They then send action potentials through large myelinated fibres of the vagus nerve to the inspiratory area in the medulla, and apneustic centre of the pons. This results in the direct inhibition of the inspiratory area, and the inhibition of the apneustic centre from activating the inspiratory area, allowing expiration to occur⁷².

RARs are myelinated fibres thought to mostly derive from the nodose ganglia. They have slower conduction velocities than SARs, with conduction velocities of over 3m/s. They are responsive to the changes in mechanical properties of lungs and airways, such as lung hyper- and hypo-inflation of the lung⁷³. They are found mostly in the larger airways, where they are thought to play a defensive role⁷⁴. A wide variety of chemical stimuli have been shown to activate RARs, however this remains controversial. Studies *in vitro* have shown that although guinea pig A δ -fibres may be excited by distilled water and hypertonic saline^{75,76}, they are insensitive to chemical stimuli such as capsaicin and bradykinin⁷⁷. However, Adcock *et al* suggested that a subset of RAR A δ fibres are responsive to capsaicin regardless of conduction velocity⁷⁸. Their stimulation has been shown to trigger a variety of defensive reflexes including cough⁶⁵.

Another class of airway sensory fibre is the unmyelinated C-fibre. C-fibres can derive from the nodose and jugular ganglia. They are not activated by mechanical stimuli such as changes in pulmonary volume, but respond to chemical stimuli, such as mediators released during lung inflammation⁶⁵. Therefore, C-fibres are considered to be the most relevant subtype of afferent fibres with respect to asthma. Based on their sites of termination, C-fibres may be classified as bronchial or pulmonary.

Bronchial C-fibres are located within the airway mucosa, and can trigger responses such as bronchoconstriction, rapid shallow breathing and mucus hypersecretion, plus the cough reflex⁶⁵. Pulmonary C-fibres are located in the lung parenchyma^{79,80}, and can cause a pulmonary chemoreflex involving bradycardia, hypotension, and apnoea followed by rapid shallow breathing^{81,82}.

C-fibres respond to a large number of chemical stimuli, including agonists of the TRPV1 (e.g. capsaicin) and TRPA1 (e.g. acrolein) ion channels, along with inflammatory mediators such as PGE₂ and bradykinin, and inhaled irritants (such as cigarette smoke and air pollution)^{78,83–86}. Studies have confirmed the expression of TRPV1 and TRPA1 receptors in mouse C-fibres using single cell polymerase chain reaction (PCR) of airway ganglia⁸⁷. It is thought that the C-fibres are activated directly by chemical stimuli, instead of indirectly as a result of bronchoconstriction. In addition to relaying sensory nerve information to the CNS, it has also been shown that the activation of C-fibres results in local axonal reflexes and the release of neuropeptides such as calcitonin gene related peptide (CGRP), Substance P (SP) and Neurokinin A (NKA)^{88,89}. Studies have shown that the peripheral release of neuropeptides can contribute to inflammatory processes in the lung that result in bronchoconstriction, mucus production and oedema⁵⁴. Therefore, when studying C-fibres and their role in innervation of the allergic asthmatic lung, both the local effects of stimulation, and the central reflex events leading to parasympathetic cholinergic effector responses must be considered.

1.3.2 Efferent pathways

It is recognised that the efferent nerves innervating the airways are mainly efferent cholinergic parasympathetic nerves. They arise in the vagal nuclei of the medulla and travel to the airways via the vagus nerve where they relay in the parasympathetic ganglia located within the airway wall⁹⁰. Acetylcholine (ACh) is the main neurotransmitter involved in parasympathetic transmission and binds to muscarinic receptors. Activation of parasympathetic pathways leads to bronchoconstriction, mucus secretion and bronchial vasodilation mediated by muscarinic M₃ receptors located on ASM and mucus glands^{66,89}. Muscarinic M₁ receptors are located in parasympathetic ganglia and in most cases appear to be excitatory. Activation of M₁ receptors results in the facilitation of cholinergic neurotransmission⁹⁰. Muscarinic M₂ receptors are located pre-junctionally on cholinergic nerve terminals and activation leads to the inhibition of acetylcholine release through decreasing cAMP to protect against bronchoconstriction as feedback autoreceptors⁹¹.

The efferent pathways of cough are different. To start with, a signal passes from respiratory bulbospinal pre-motor neurons to laryngeal motor neurons. This then causes a large contraction of the abdominal muscles and larynx. Consequently the air is expelled, resulting in the characteristic

cough sound^{92,93}. These motor neurons are present in intercostal, diaphragm, abdominal and glottal muscles^{94,95}.

1.3.3 Airway sensory nerves and the LAR

The aetiology of the LAR has been closely associated with the activation of airway sensory nerves. Studies in the Respiratory Pharmacology group suggest that a cholinergic nerve reflex leads to LAR²⁷ (Figure 1.2). Raemdonck *et al* showed that in a conscious OVA rat model, LAR occurred 5 hours later from EAR, with Th2 eosinophilic inflammation and responsive to gold standard therapies. The LAR response was abolished under anaesthesia, and ruthenium red, a non-selective TRP channel antagonist attenuated the response, suggesting a role for airway sensory nerves. In addition, tiotropium, a long-acting muscarinic antagonist was also shown to inhibit the response, indicating that the LAR is driven by a parasympathetic reflex. Taken together this suggests that activation of airway sensory nerves leads to a signal being sent to the CNS which results in a cholinergic bronchoconstrictor response. This finding also translates to asthmatic patients in the clinic, where Long Acting Muscarinic Antagonists (LAMAs) can improve symptoms and lung function⁹⁶. In addition, a member of the Transient Receptor family of ion channels – TRPA1 was also shown to attenuate the response, suggesting a possible mechanism for activation, more information on TRPA1 can be found in the following section.

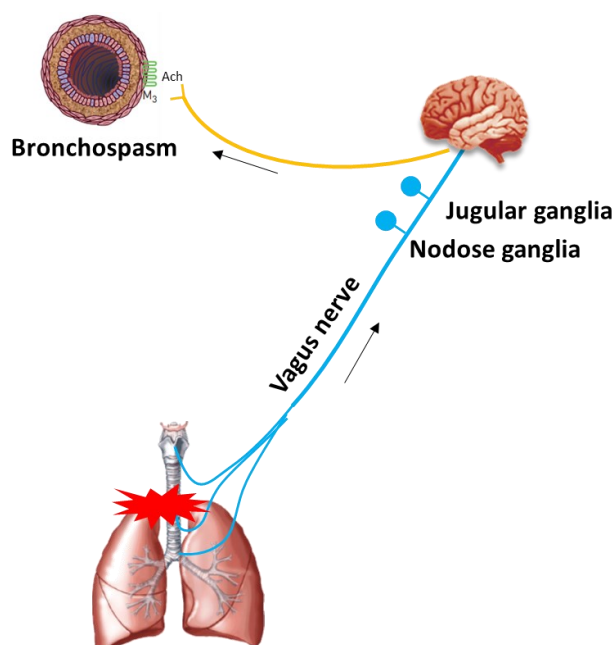


Figure 1.1 Schematic representation of the proposed mechanisms involved in the late asthmatic response (LAR).

The hypothesis is that allergen challenge or other stimuli lead to sensory nerve activation via central reflex events and ultimately a parasympathetic cholinergic bronchoconstrictor response. This is mediated by the activation of muscarinic M₃ receptors by acetylcholine (Ach), causing bronchospasm.

1.3.4 Airway sensory nerves and the cough reflex

The cough reflex response consists of three parts: first, the initiation of the reflex through sensory nerve input; second, a complex central neural network to process information; third, stimulating the efferent limbs and motor output to deliver a cough⁵⁴. Human and animal coughs can be caused by various stimuli such as citric acid^{58,97}, capsaicin^{98,99}, bradykinin¹⁰⁰, and prostaglandins¹⁰¹. Other causes include airway obstruction, mechanical irritation, dust, cigarette smoke, chemical fumes, changes in ion concentration, or osmotic pressure of airway surface fluids^{100,102,103}. These stimuli trigger receptors in or under the airway epithelium. Stimulation of these peripheral sensory nerve terminals produces afferent fibres, which are innervated by the vagus nerve, and thereafter transmit information to specific areas in the brainstem. The exact mechanism of this area is not yet determined¹⁰⁴. Subsequently, the reflex stimulation of the efferent limbs leads to the introduction of somatosensory nerves, causing the contraction of the skeletal muscles in abdomen and chest, and results in coughing⁵⁴.

1.4 Pre-clinical model of the LAR and cough

Models of diseases are required to investigate mechanisms and novel therapeutics. Animal models are essential tools to help researchers probe disease mechanisms and discover new asthma drugs. It is also important to use these models to investigate existing therapeutics to fulfil their mechanisms of action.

1.4.1 Animal models of the LAR

Although there are both allergic and non-allergic variants of asthma, the majority of *in vivo* models are based on the allergic form. Over the past 50 years, a standardised allergen inhalation challenge model has been established⁷. This thesis will therefore introduce two of the most commonly used experimental models of allergic asthma - the ovalbumin (OVA) and house dust mite (HDM) models.

1.4.1.1 Ovalbumin Model

OVA is a protein extracted from chicken egg white and for decades has been used to induce allergic response in rodents in classical models of allergic asthma. In doing this, all protocols involve a sensitisation stage and a challenge stage. In the sensitisation stage, animals are usually sensitised with OVA combined with an adjuvant systemically such as by intraperitoneal (i.p.) or subcutaneous (s.c.)

route of administration. The adjuvant, such as AlumTM or aluminium hydroxide, can prime the immune system towards a Th2 phenotype¹⁰⁵. Sensitisation typically requires two or more sequential systemic doses, separated by a specific amount of time. In the challenge stage, animals are challenged by OVA via a topical dose (i.e. intranasal, aerosol or intratracheal). Slight model variations including differences in doses, sensitisation time, and challenge times have been established by different research teams.

The OVA model can successfully mimic key features of asthma. Sensitisation leads to an increased total and OVA-specific IgE levels¹⁰⁶ and following this, the model can induce key asthma symptoms such as EAR and LAR, non-specific AHR and oedema¹⁰⁷. In addition, the induced key features can be modulated by gold standard therapies such as glucocorticoids and β_2 -adrenoreceptor agonists¹⁰⁸. Therefore, the OVA model has been used as a representative platform for the investigation of novel therapeutics. The OVA model has been established in guinea pigs, rats and mice. Brown-Norway rat and BALB/C mouse has become the most widely used strains¹⁰⁹. C57/BL6 mice are also regularly used for the advantage of readily available genetically modified lines.

However it is of note that this model has its limitations: Firstly OVA is rarely an antigen for humans to develop asthma to and as such it is not clinically relevant. Secondly the administration route of sensitisation does not mimic the topical routes which is thought to be how humans develop sensitisation clinically¹¹⁰. Thirdly, it has been reported that OVA has tolerance issues when used to induce allergic response in long term studies. When inducing chronic models, suppression of eosinophilia, AHR and OVA-specific IgE have been demonstrated¹¹¹. However, despite these limitations, a well characterised, reproducible LAR can be monitored utilising OVA, and so this is the model I have utilised throughout my thesis.

1.4.1.2 House Dust Mite Model

Due to the limitation above, models with more disease relevant allergens such as house dust mite (HDM) have also been developed. In almost 80% of asthmatic patients, HDM has shown to be a relevant allergen¹¹². It has also been shown to be related to some other allergic disorders such as allergic rhinitis and atopic dermatitis¹¹³. The mostly common used HDM strains are *Dermatophagoides pteronyssinus* (*Der p*) and *Dermatophagoides farina* (*Der f*)¹¹⁴. Usually multiple topical instillations are performed to induce HDM models, unlike the OVA which requires different mode of administration for sensitisation and challenge stages. The advantage of HDM model is that firstly, as said before, HDM is a more clinically relevant allergen. Secondly, no adjuvant or systemic sensitisation is needed as animals can be sensitised to HDM via the respiratory mucosa exposure. Thirdly eosinophilic

inflammation is sustained following chronic exposure^{115,116}. However, unlike the OVA model, HDM model has not been fully studied and the role of immune cell populations and inflammatory mediators still needs to be investigated. A study has also shown that asthma-like airway inflammation can be caused by a single topical dose of HDM, resulting in a suggestion that initial inflammation in this model is innate in nature¹¹⁷. Furthermore, none of these recently developed HDM-driven models display an EAR or LAR. Consequently, HDM driven model was not used in this thesis.

1.4.2 Animal models of cough

Reflex cough occurs following the activation of airway sensory nerves. Therefore, in order to understand the neural components of the cough reflex, we adopted laboratory-based experimental methods. The use of animal models with enhanced cough reflex response and basic pathology is crucial for understanding the underlying mechanism of chronic cough and may prove to be of great significance in identifying and verifying new, effective and safe antitussive therapies. Some species including guinea pigs, dogs, cats, rabbits, mice and rats have been used to establish animal models to study cough reflexes^{118–122}. Among these species, some believe that the anatomy and physiological structure of guinea pigs are most similar to humans. The lungs have a similar neuroanatomy to man⁷¹, and in addition, guinea pigs have similar sensitivities to tussive stimuli capsaicin and citric acid¹²³. Therefore, guinea pigs are used in this thesis as they provide the most suitable model system that can be used for preclinical studies of cough reflex in *in vitro* and *in vivo* systems¹²⁴.

First, neurons can be isolated from guinea pig vagal ganglia. Once isolated, intracellular calcium levels monitored in the presence of agonists and antagonists can be investigated as markers of neuron activation¹²⁵. The cells can also be used for targeted expression at the mRNA level⁸⁷. Secondly, the vagus nerve can also be isolated to study the effect of anti-tussive drugs or tussive stimuli. The nerve depolarisation is recorded as a measure of activation. It has been shown that in this system, compounds that induce isolated depolarisation can also induce a cough response in the body^{122,126,127}.

One of the studies to be conducted as *in vivo* models is by using single fibres of the vagus nerve to study the effect of pro-tussive or antitussive compounds on the action potential firing of airway specific afferent fibres¹²⁸. It is physiologically meaningful to study the effect of these compounds on nerve endings in the entire animal body. Finally, conscious guinea pigs can be used as a model system to study cough response. This involves placing the guinea pig in a separate Perspex chamber and measuring the effect of the aerosolised compound on the cough reflex. Studies have shown that cough responses in guinea pigs are very similar to human cough responses^{83,129,130}.

1.5 Ion channels implicated in airway reflexes

1.5.1 Overview

The initiation of a number of protective airway reflexes, including cough and reflex bronchoconstriction are associated with the activation of a range of ion channels expressed on airway sensory nerve terminals. The Transient Receptor Potential (TRP) family of ion channels are one such example of cellular sensors in the airways that are activated by a wide range of mechanical, thermal and chemical stimuli^{131,132}. They were originally discovered in the eye of the *Drosophila melanogaster* fly and so named for their transient response to bright light. Following this initial discovery a group of homologues have been discovered, forming a large protein family¹³³. There are 30 mammalian TRP subunits, classified into seven subfamilies, based on sequence homology, including TRPA (ankyrin), TRPV (vanilloid), TRPM (melastatin), TRPC (canonical), TRPP (polycystin), TRPML (mucolipin), and TRPN (NOMP-C)¹³².

The TRP family comprises of cation-selective ion channels which show a preference to calcium ions, and as they conduct cations they are capable of depolarising cells¹³⁴. TRP ion channels are composed of six transmembrane-spanning domains with the channel pore. They are located between transmembrane domains 5 and 6, intracellular C and N termini, and different levels of ankyrin repeats¹³⁵. They are broadly expressed throughout the body. TRP channels are widely expressed throughout the body, but in particular TRPA1, TRPV1, TRPV4, TRPM8 and TRPM3 have been identified in the respiratory tract and have been shown to activate airway sensory nerves and potentially contribute to reflex events^{136,137}.

1.5.2 TRPA1

In this thesis, the focus of my investigation is the TRPA1 channel. TRPA1 is the only member of the ankyrin subfamily that has been found to be expressed in mammals. TRPA1 was originally discovered in human cultured fibroblasts¹³⁸, and was named because on its N-terminal domain there are a large number of ankyrin repeats¹³⁹. The human TRPA1 gene includes 29 exons, composed of 73,635 base pairs located in chromosome 8 band q21.11¹³¹.

TRPA1 is a calcium-permeable voltage-dependent cation channel. TRPA1 is expressed in neuronal cells such as the spinal dorsal root ganglia, nasal trigeminal, and vagal airway neurons^{139,140,141}; and non-neuronal cells such as human lung fibroblast cells and pulmonary alveolar epithelial cells in the respiratory tract¹⁴².

TRPA1 channels are chemosensory and can be activated by a wide range of chemical stimuli, including natural substances, such as cinnamaldehyde, allicin and cannabinol^{143,144}, environmental irritants,

such as diesel exhaust particles (DEP) and acrolein^{145,83} and products of oxidation, such as 4-oxynonenal and 4-hydroxynonenal^{146,147}. In addition to chemical ligands, there is evidence that TRPA1 channels are activated by noxious cold temperatures below 17°C¹³⁹. TRPA1 channel activation by cold temperatures could be due to a conformational transition (or denaturation) of the channel protein structure¹³². It was also reported that TRPA1 plays a role in GPCR signalling in sensory nerves. PGE₂ and bradykinin following activation of their GPCRs (EP₃ receptor and B₂ receptor respectively) can lead to activation of TRPA1¹⁴⁸.

TRPA1 activation by electrophilic compounds, such as acrolein and cinnamaldehyde, is via either conjugate or direct addition of cysteine residues to the N-hydroxysuccinyl esters¹⁴⁹. The chemistry behind activation has not been fully elucidated as there is some controversy for each compounds¹⁴⁸. TRPA1 activation by products of oxidation is also in a complex manner. TRPA1 is well known as a sensor of oxidative stress¹⁵⁰. For example, glutathione-sensitive oxidation of cysteine residues C633 and C856 located in the N-terminus of the channel can mediate a stimulatory action of hyperoxia¹⁵¹. Generally, most of ROS (reactive oxygen species), RNS (reactive nitrogen species) and RCS (reactive carbonyl species) act as TRPA1 activators¹⁵². For example, the typical ROS, H₂O₂ is known to be a TRPA1 activator through its accumulation within cells from various industrial products¹⁴⁸. The environmental irritant DEP was found to activate TRPA1 indirectly through the activation of the aryl hydrocarbon receptor (AHR) by polyaromatic hydrocarbons, which are a major constituent of DEP. Activation of AHR then leads to mitochondrial ROS production, which subsequently activates TRPA1¹⁴⁵.

The whole respiratory tract is innervated by primary sensory afferent nerves, which can be activated by mechanical and chemical stimuli via activation of TRPA1 on these vagal sensory afferents¹⁵³. Unlike other TRP channels; TRPA1 only seems to activate C-fibres, where its activation can potentially trigger airway reflexes⁷⁸.

TRPA1 activation has been linked to increased bronchoconstriction. In allergic asthma, exposure to certain antigens could trigger an EAR. In some subjects, this is followed by a LAR. The LAR is thought to be one of the defining symptoms of asthma, and it has widespread use in the clinical assessment of new therapeutic entities. The underlying mechanism of the LAR, however, remains unclear. It was reported in studies on rodents, that TRPA1 activated vagal bronchopulmonary C-fibres in the lung, and induced a LAR in sensitised rodents following allergen challenge²⁷. The likely mechanism is that allergens stimulate the channel indirectly via the release of endogenously produced TRPA1 activators. Figure 1.3 shows a schematic of a TRPA1-driven sensory nerve activation, and a parasympathetic, cholinergic reflex bronchoconstrictor response which may be responsible for the LAR²⁷. In addition, TRPA1 activators such as toluene di-isocyanate, can induce respiratory symptoms including LAR in

exposed workers¹⁵⁴, and it was also suggested that this irritant may initiate a LAR directly by stimulating sensory nerves¹⁵⁵. These data further suggest that neuronal reflexes may be a key driver of the LAR.

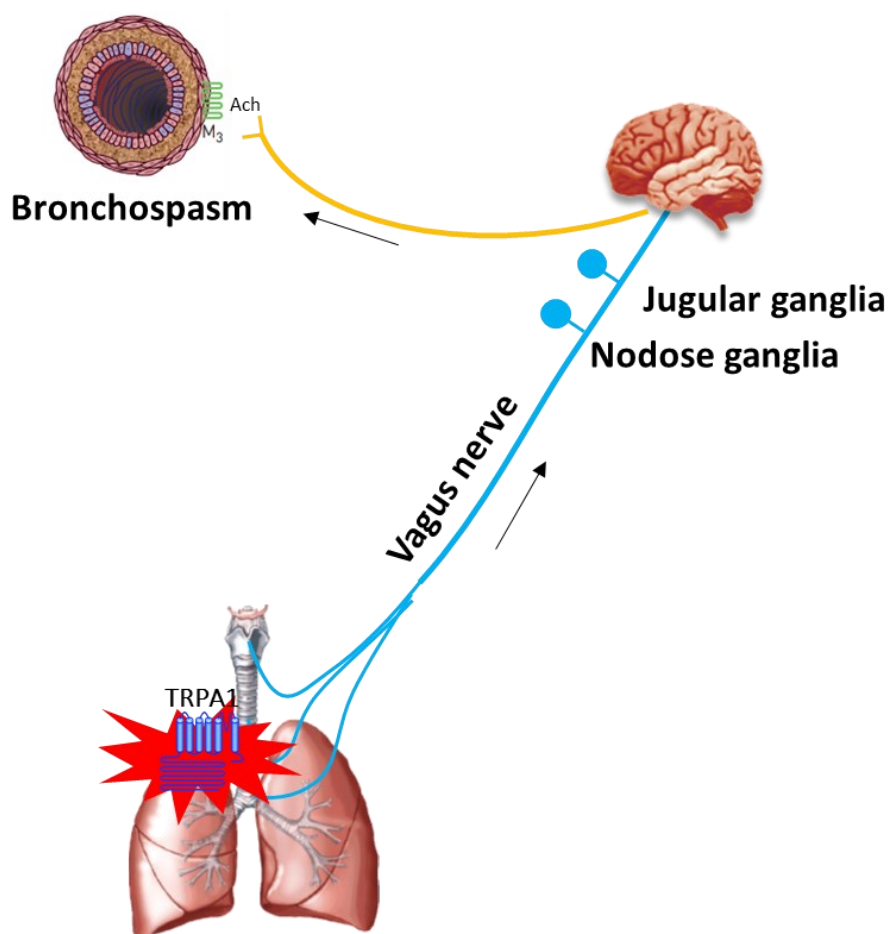


Figure 1.2 Schematic representation of the proposed mechanisms involved in the late asthmatic response (LAR).

The hypothesis is that allergen challenge or other stimuli lead to sensory nerve activation via the TRPA1 channel (blocked by TRPA1 antagonists) and central reflex events (blocked by anaesthesia), ultimately leading to a parasympathetic cholinergic bronchoconstrictor response. This is mediated by acetylcholine (ACh) activating muscarinic M₃ receptors, causing bronchospasm (blocked by anticholinergics).

Along with inducing reflex bronchoconstriction, activation of TRPA1 channels has also been reported to stimulate vagal nerves to induce cough. A range of TRPA1 agonists can stimulate vagal nerve fibres¹⁵⁶ and trigger cough in animals and man^{83,157}. In a conscious guinea pig model, TRPA1 antagonists have also been shown to inhibit cough in response to the clinical challenge agent citric acid^{158,159}. The TRPA1 agonist cinnamaldehyde evoked cough in clinical studies in healthy volunteers¹²⁶. In addition to causing cough, it has also been postulated that cough is worsened by a variety of TRPA1 agonists in patients of chronic cough¹⁶⁰. Together with the evidence linking TRPA1 to the LAR, this

would suggest that TRPA1 is a key ion channel in the initiation of symptoms such as cough and parasympathetic bronchoconstriction, which are upregulated in a number of airway diseases. Therefore, targeting the TRPA1 channel could lead to novel, safe and effective therapies.

1.6 Disodium cromoglycate (DSCG)

1.6.1 History of the discovery of DSCG and overview

DSCG was discovered in 1968 by Dr Roger Altounyan (1922-1987), an Anglo-Armenian physician and pharmacologist. Although he pioneered the discovery of drug which saved thousands of lives, he gave credit for the discovery of this drug to the ancient Egyptians (3000 BC). When he was investigating certain plants and herbs which have bronchodilation properties, he found that ancient Egyptians noticed that a soup made with a weed called khella relieved spasms and helped people breathe better. Dr Altounyan and the team studied the key extract – Khellin and synthesised a series of derivatives¹⁶¹. During this period of time, Dr Altounyan who was himself a lifelong asthma sufferer tested the effect of compounds on himself and discovered a compound called FPL 670. Although FPL 670 did not stop the asthma attacks he was getting after inhaling the allergen triggering them, it prevented attacks when he was taking the drug before breathing the allergens. These promising initial results were confirmed in clinical trials and FPL 670, now known as disodium cromoglycate or DSCG, became available for use in asthma treatment in the United Kingdom in 1968¹⁶².



Figure 1.3 Khella (*Ammi visnaga*).

The drug was first manufactured and sold by the firm Fisons Pharmaceuticals for the prophylaxis of asthma under the name Intal. Since then the drug has been rebranded many times and is also known as Cromolyn, Cromoglicic Acid, Cromogloz, Gastrocrom, Inostral, NasalCrom and Opticrom.

As opposed to classical drug development procedure, DSCG was first tested in humans and was only later further investigated in animal models. So far DSCG has rarely reported adverse effects. More impressively no adverse drug interactions have been reported as yet¹⁶³. As Cromoglycate is a highly

ionized water-soluble compound, it does not cross cell membranes and can be effectively administered only by inhalation¹⁶⁴.

The use and understanding of DSCG developed in parallel with the growing understanding of asthma pathogenesis. Research performed over the past few decades has shown the anti-allergic and anti-inflammatory effect of DSCG is via the inhibition of IgE-stimulated mediator release from mast cells. It prevents mast cell activation and release of inflammatory mediators by degranulation by inhibiting chloride transport and protein kinase C¹⁶⁵. However, DSCG has also been shown to have a number of other beneficial effects which cannot be fully explained by this mechanism.

1.6.2 Historical research on DSCG

Among the earliest work is a clinical study performed by Dr. Altounyan, which showed that when administered prior to allergen exposure, DSCG could prevent the allergen induced early asthmatic response (EAR)¹⁶⁶. In addition, DSCG was not only shown to inhibit EAR, but has demonstrated efficacy against the LAR^{14,24,167,168}. Booij-Noord et al. demonstrated the effectiveness of DSCG in inhibiting house dust mite induced EAR and LAR in 1971¹⁴. Later work has confirmed this and has in addition shown the effectiveness of DSCG against airway hyper responsiveness (AHR) to spasmogens such as histamine and methacholine without an immediate bronchodilator effect^{24,167}.

The timing of DSCG administration is essential. Delivery too early before allergen exposure could be ineffective and DSCG after the EAR, but before the LAR is ineffective in preventing LAR and post allergen AHR. This not only highlights a short half-life, but could suggest that the LAR following allergen exposure could result from indirect or direct mediator release following mast cell degranulation¹⁶⁹.

Analysis of human lung tissue showed that its effect on functional outcomes such as the EAR was accompanied/associated with a reduction in mast cell degranulation¹⁷⁰. DSCG has since been shown to be capable of stabilising the mast cell membrane and thus block the release of inflammatory mediators such as histamine, leukotrienes and 5-HT. Later investigations using isolated human/animal cells and bronchoalveolar lavage (BAL) fluid have demonstrated that DSCG can block the activation of many other inflammatory cells such as eosinophils, T-lymphocytes, neutrophils, monocytes and reduces allergen specific IgE in BAL fluid^{171–173}. DSCG possibly has this effect on inflammatory cells by reducing the expression of adhesion molecules and cytokines^{172,174}. In fact, many other anti-inflammatory effects of cromoglycate-mimic drugs that have been reported include inhibition of leukocyte activation and inhibition of eicosanoid and cytokine release from different cell types¹⁷⁵. Inhalation of cromolyn sodium had no effect bronchodilator effect in any of the reported studies.

Animal models in a variety of species have demonstrated that DSCG can inhibit both the EAR and LAR^{176–178} and can inhibit experimentally induced cough in guinea pigs¹⁷⁹. One report compares nedocromyl sodium, a different cromone, and DSCG in citric acid induced cough in dogs. The author found that nedocromyl but not DSCG was capable of inhibiting CA induced cough in dogs. The same concentration was used for both drugs, even though a lower potency has been reported for DSCG¹⁸⁰. A higher dose of DSCG therefore could have inhibited the cough. In asthmatic children DSCG was found to reduce daytime and night-time cough¹⁸¹.

A review demonstrated an observation that DSCG can have an inhibitory effect on AHR regardless of allergic status. This highlights that not all of DSCG functions stem from its effect on mast cell stabilisation¹⁸². Indeed there is more evidence regarding DSCG have been reported that would suggest an activity outside of the expected IgE - mast cell degranulation effect. It has been shown that DSCG is effective against bronchoconstriction thought to be unrelated to mast cell degranulation. This includes bronchoconstriction induced by stimuli such as exercise, SO₂, bradykinin, cold air, distilled water^{183,184}. Moreover newly developed mast cell stabilisers have been shown to have little or no effect in the clinic¹⁸⁵.

Following results in experimental animal models demonstrating the ability to modulate sensory nerve function, it has been suggested that the effect of cromones, such as DSCG, on bronchoconstriction in response to non-IgE mediated stimuli is to be through an effect on sensory nerves¹⁸⁶. Aerosolised DSCG can reverse and prevent reflex bronchoconstriction in anaesthetised dogs and it has been suggested to be related to suppression of lung irritant receptor activity¹⁸⁷. However electrical stimulation of the vagus nerve induced an increase in resistance that was not inhibited by DSCG, again highlighting a possible effect of DSCG on lung irritant receptors. Jackson et al. have also reported that DSCG causes a reflex hypotension and bradycardia which is mediated via the vagus nerve when given intravenously to the anaesthetized dog¹⁸⁷. Dixon et al. showed that the hypotension caused by intravenous injection of DSCG in anaesthetised dogs was prevented by lignocaine instillation into the pericardium and that it also prevented sodium cromoglycate from reversing reflex bronchoconstriction induced by inhalation of an aerosol of histamine. They suggested that sodium cromoglycate produced a reflex hypotension by acting on nerve receptors located in the endocardium and, regarding bronchoconstriction that it reversed histamine-induced reflex bronchoconstriction by activating neuronal receptors in the left ventricle of the heart¹⁸⁸. The authors postulated that the reduction of bronchoconstriction could be mediated by a direct DSCG effect on afferent sensory nerve endings located within the endocardium, suggesting that it is possible for inhaled DSCG to be absorbed

in the lung at sufficient levels to reach the left ventricle. It was also reported that DSCG is capable of inhibiting the activation of pulmonary C-fibres by 5-HT¹⁸⁹.

In the clinic DSCG can be used to block ACE-inhibitor induced cough¹⁹⁰. Most likely the effect of DSCG is through an inhibition of bradykinin and substance P and their effect on C-fibres as these pro-tussive mediators are normally degraded by ACE. In 2017, a novel formulation of DSCG was shown to reduce coughing in patients with IPF⁵⁰. Although the mechanism was not studied.

1.6.3 Potential mechanisms of the beneficial effects of DSCG

Previous work has been done mostly in human/animal objects, tissues or cells. With the development of molecular biology, more recent work has demonstrated that DSCG is a potent agonist of the G-protein-coupled receptor 35 (GPR35)^{191–193}, which provides a brand new angle to explain its effect. GPR35 is a G protein-coupled receptor that is encoded by the GPR35 gene in humans. G protein-coupled receptors are one of the biggest cell surface protein families composed of over 1000 members and making 3% of the human genome. It is known that GPCRs are involved in the mechanism of action of a wide range of therapeutics¹⁹⁴. Although ligands have been identified for many GPCRs there are still more than 100 GPCRs with no endogenous ligand known as yet and which are called orphan receptors. Orphan receptors may provide opportunities for drug discovery as they could be involved in signaling pathways and disease control/progression. The GPR35 receptor is one of these orphan receptors which are not well characterised.

As GPR35 is expressed on many immune cells¹⁹¹ and appears to be upregulated following IgE challenge, it is possible that DSCG reduces the inflammatory response by dampening this interaction. GPR35 is coupled to the Gi signalling pathway and it was shown in an experiment in rat peritoneal mast cells that Gi pathway involves the release of Ca²⁺ from internal stores¹⁹⁵. As such, Gi coupled receptor activation of IP3K/PLC and FcεRI could be a possible mechanism by which the signals induced by these receptors cause a synergistic enhancement of mast cell degranulation¹⁹⁶. This could help explain its actions on mast cell degranulation.

In addition GPR35 is found to be expressed on rat dorsal root ganglia and agonist of GPR35 can block N-type calcium channels¹⁹⁴ and activation of GPR35 produces antinociception in animal models of pain, indicating a possible role in sensory nerves. This potential mechanism that DSCG might have its link to sensory nerve via GPR35 is indeed the focus of this thesis.

Last but not least, DSCG also inhibits the assembly of an active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the neutrophil, thereby preventing tissue damage induced by oxygen

radicals¹⁹⁷. DSCG was also reported as anti-oxidant when studying myocardial reperfusion in rat hearts¹⁹⁸.

1.7 Outline of thesis

Asthma is a disease with increasing global prevalence and patients typically suffer from symptoms such as wheezing, breathlessness, chest tightness and cough. Although in most cases asthma can be well controlled with current treatments, there remains an unmet need as patients do not always comply with therapies, and some remain unresponsive to treatment. In allergic asthma, exposure to allergen can lead to episodes of bronchoconstriction known as the late asthmatic response (LAR), a characteristic feature of asthma which has been shown to involve the activation of airway sensory nerves in a preclinical model. In asthmatics, DSCG is known to modulate the LAR but its use is limited because of its short duration of action seen evidenced by its pharmacokinetic profile. Although a safe and effective drug, the mechanism of action of DSCG remains unclear. The overall aim of this thesis is to investigate the mechanism with the possibility to develop a novel therapy with an improved PK profile and thus be a more useful treatment.

1.7.1 Hypothesis

The hypothesis that formed the basis of this project was that DSCG can inhibit airway sensory nerve driven reflex responses such as the LAR and cough.

1.7.2 Structure of investigation

The aims of this thesis are to:

1. Investigate the impact of DSCG on a pre-clinical rodent model of the LAR as well as measure the impact of DSCG on TRPA1-driven stimulation of sensory nerve activation and *in vivo* cough responses (Chapter 3).
2. Investigate the involvement of GPR35 in the inhibitory effect of DSCG on airway sensory nerve activation and the LAR (Chapter 4).
3. Examine the effect of a selective GPR35 agonist on airway sensory nerves and the LAR (Chapter 5).
4. Examine the effects of DSCG and selective GPR35 agonism on a wide range of TRPA1 activators-driven nerve activities (Chapter 6).

Chapter 2 Methodology

2.1 Introduction

This chapter describes the general methodology for the experimental techniques presented in this thesis. In this section the methodology is described in an order to best explain the utility of each technique. Note that the sequence the techniques are presented in here does not reflect the order in which they are presented in results chapters. Rather, in each results chapter the techniques are presented in the order which they were carried out for the process of hypothesis-driven investigation. Additional specific details regarding each experimental protocol and statistical analysis are described in the respective methods section of each results chapter.

In order to investigate complex *in vivo* phenomena such as cough and the late asthmatic response, it is important to use a range of experimental techniques such as those described here to examine individual components of the afferent nervous system. Therefore in this section the range of techniques presented goes from *in vitro* techniques to examine the gene expression and functional responses of airway neurons and vagus nerves, through to *in vivo* techniques to examine the action potential firing of nerves and whole body responses such as cough and the late asthmatic responses. It is not ethical to perform these techniques on humans, therefore animal models are used here. The most suitable animal models were selected to best represent the human situation. Guinea pigs are the smallest rodent species with an audible cough response, and a rat model is used as a surrogate for the clinically observed late asthmatic response. All the techniques presented used primary cells and tissues in order to best mimic the *in vivo* function of those cells/tissues, and human vagus nerve tissue was used in the whole vagus nerve tissue assay (*Section 2.4*) in order to examine whether the function and pharmacology observed in rat and guinea pig tissues was translational to humans.

2.2 Biological material: Animals and Human tissues

All animal experiment procedures were performed under UK Home Office Guidelines based on the Animal (Scientific Procedures) Act of 1986 and according to ARRIVE guidelines¹⁹⁹ under project licence number 70/8845.

2.2.1 Animals

Male Brown Norway Rats were purchased from Charles River Laboratories (Germany) (7 weeks; 225-250 grams at purchase) and Male Dunkin-Hartley guinea pigs were purchased from B&K laboratories (UK) (4 weeks, 300-400 grams at purchase). Animals were left to acclimatise for at least 7 days at 21°C

with food and water freely available prior to any experimental procedures. All animal procedures were performed in accordance with a Home Office Project license by trained Personal License holders.

2.2.2 Human tissue

Donor lungs unsuitable for transplant were supplied by International Institute for the Advancement of Medicine (IIAM; New Jersey, US). Ethical approval for human tissue use was obtained from the Royal Brompton and Harefield Trust. Whole lung “en-bloc” with vagal nerves attached were received from IIAM and the vagus nerves were carefully removed and dissected for further experiments²⁰⁰.

2.3 Expression and function of isolated airway-terminating neurons

In this section, the methods used to identify individual airway-terminating neurons and examine their gene expression and function *in vitro* are described. These neurons are essential for detecting stimuli in the lungs, and they convey signals initiated in the airways along their axons to the central nervous system in order to control respiratory reflexes such as cough and bronchospasm (Section 1.6 and 2.3).

The jugular and nodose ganglia of the vagus nerves are where the cell bodies of airway-innervating sensory nerves are housed. Removal of the ganglia and enzymatic digestion can be used to isolate individual jugular and nodose ganglia neurons. However, these vagal ganglia house neurons that innervate all of the internal visceral organs, as well as the airways. Therefore in order to investigate the expression and function of airway-terminating neurons, rat and guinea pig airway sensory neurons were stained *in vivo* with a tracer dye taken up by airway neurons. Following a minimum 12 days period to allow the dye to reach the vagal ganglia, animals were sacrificed, and vagal ganglia removed. The isolated airway neurons could then be identified by fluorescent imaging of the stain and used for examination of gene expression, or functional responsiveness as monitored by intracellular calcium or oxidative stress imaging studies¹²⁵.

2.3.1 Staining of airway-terminating neurons

The animals were intranasally (i.n.) dosed with 1mL/kg of retrograde neuronal tracer Dil (DiI_{C18} (3) (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate; 0.625mg/ml in 0.9% sterile saline with 2% ethanol, v/v). Dosing with Dil was performed 14 days prior to experimentation to allow the retrograde tracer dye to move laterally along the axonal membranes of the neurons innervating the airways up to the cell bodies of the neurons located in the vagal ganglia²⁰¹. 14 days after the procedure the animal were sacrificed by overdose of pentobarbitone (200 mg/kg, i.p.), and the vagal ganglia were collected for sensory neuron isolation¹²⁵.

2.3.2 Harvesting and dissociation of vagal ganglia neurons

All procedures were performed in sterile condition with sterilised (autoclave/irradiation) tools and filter-sterilised solutions. The enzymatic isolation of the cells was done in a sterile biological cabinet. The animals were sacrificed with pentobarbitone (200mg/kg injected intraperitoneally). The head and neck were doused with 70% (v/v) ethanol for cleaning and sterilisation. The skin was removed followed by the top of the skull and neck muscles. The dorsal part of the mandible was then carefully cut away taking care to avoid damaging underlying soft tissue (Figure 2.1).

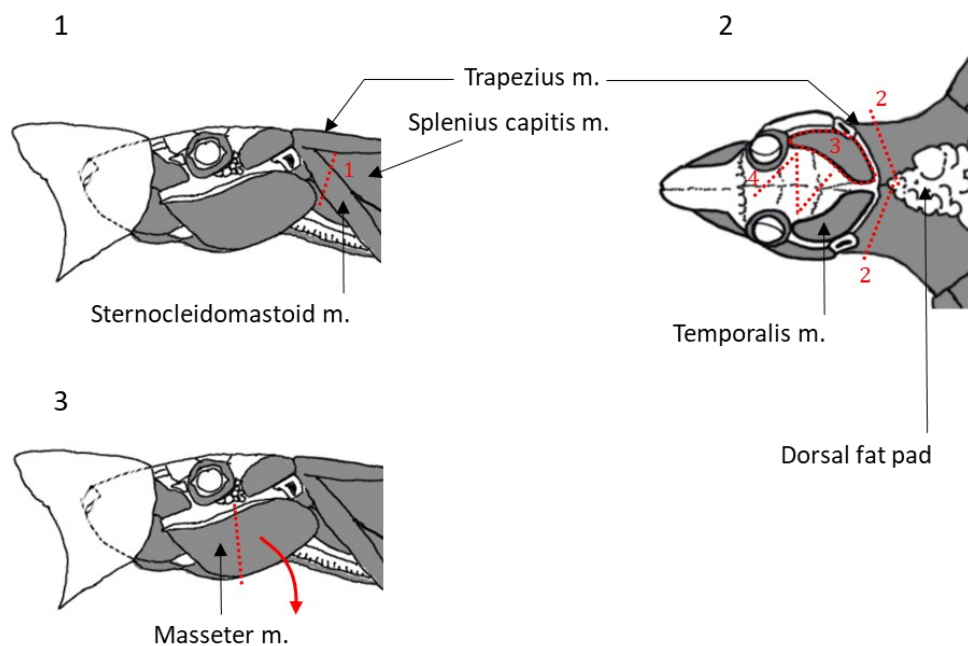


Figure 2.1 Schematic showing the process for excising the neck and head skin and muscles

As shown below (Figure 2.2A), the nodose and jugular ganglia are located behind the ear bone (tympanic bulla) which need to be removed first in order to access the ganglia. To do so the bones located around the ear bone were removed (Figure 2.2B). The brain's cervical lobes and cerebellum were then excised, taking care not to pull on the spinal cord, intracranial nerves (optical and auditory) and cranial nerves (nerves IX or glossopharyngeal, X or vagus, and XI or accessory) which were cut. Once the nerves were cut and the ear bone connection to the skull severed, the ear bone was carefully removed to reveal the underlying soft tissue containing the vagal nodose and jugular ganglia. The muscles were gently pulled away to reveal the jugular ganglia, the nodose ganglia (in case of the guinea pig) and the start of the vagus nerve (Figure 2.2C). In the case of the Brown Norway Rat, the anatomy was slightly different (Figure 2.2D) as there is only one vagal ganglia which contain both types of neurons but the extraction process remains the same.

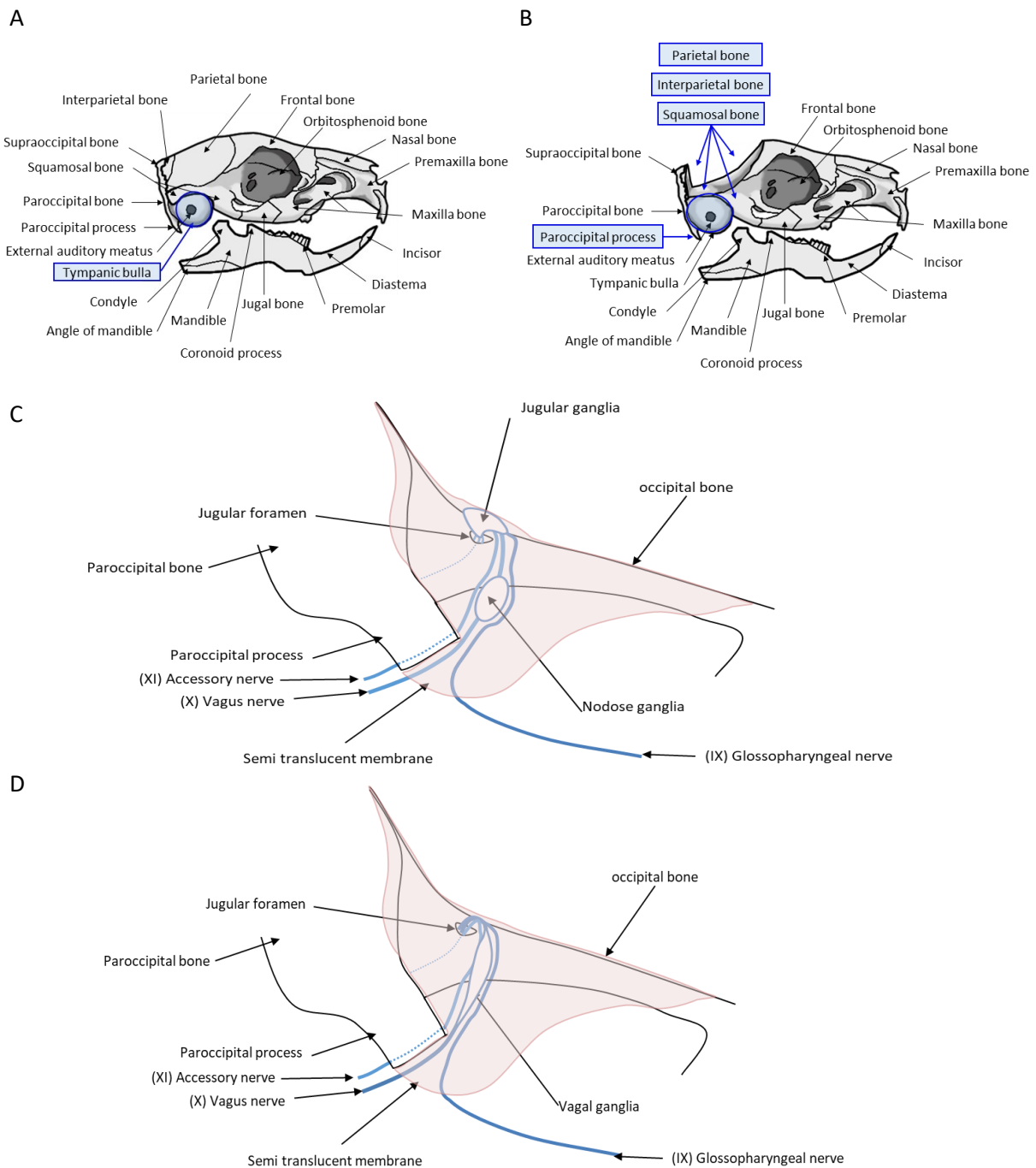


Figure 2.2 Schematic showing the process for excising the neck and head skin and muscles

A: Schematic of the guinea pig skull once skin and muscles have been removed. B: Schematic of the guinea pig skull once the top on the skull has been removed, the brain and cerebellum excised and support to the ear bone removed. C & D: Close up schematic of the jugular and nodose ganglia in the guinea pig (C) and vagal ganglia in rat (D) as seen once the ear bone has been removed.

Once exposed the ganglia were gently dissected free of most of the connective tissue holding them to the bone structure and adjacent nerves. The extracted ganglia were then placed under a dissection microscope and the ganglia were cleaned of their surrounding connective tissue and nerves. The

dissection was different between rats and guinea pigs because the guinea pigs have separated jugular ganglia and nodose ganglia whereas they are both conjoined together into a vagal ganglia for the rat (Figure 2.3).

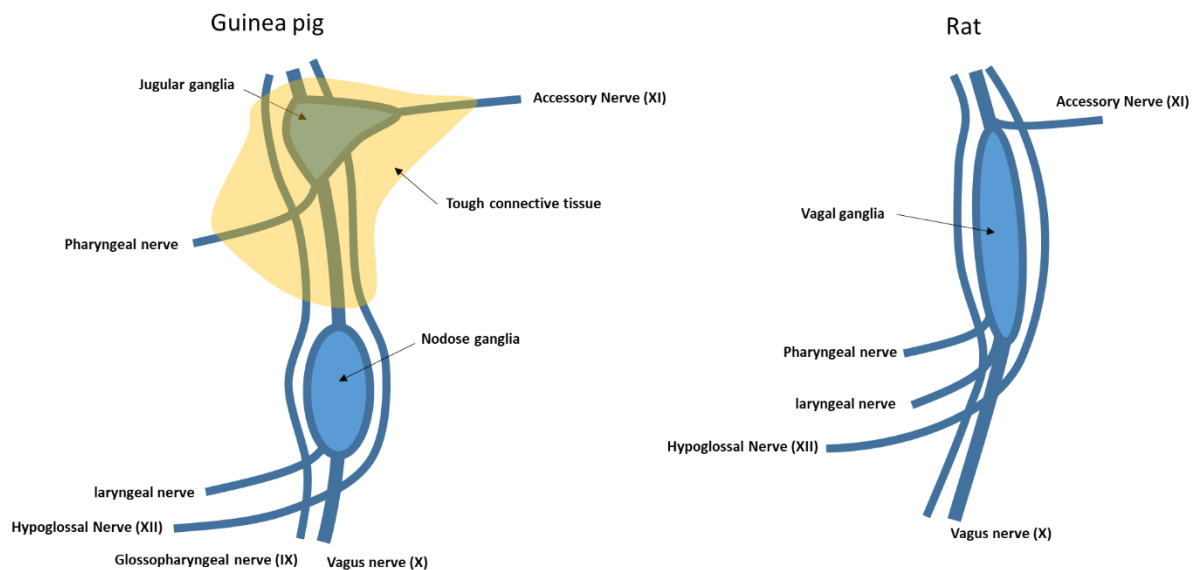


Figure 2.3 Schematic of the disposition of the vagal ganglia and surrounding nerves for the guinea pig (left panel) and the rat (right panel)

To isolate the sensory neurons, the ganglia were enzymatically dissociated. The ganglia were first incubated with collagenase type IV for 15 minutes in presence of 300 μ M Calcium chloride at 37°C to remove tough enclosing connective tissues. The enzymatic reaction was stopped by immersion in ice cold calcium free HBSS. The ganglia were then successively incubated with activated papain in presence of 300 μ M Calcium chloride (30 minutes, 37°C) then collagenase type IV in calcium free solution (45 minutes, 37°C). After each incubation, ganglia were collected by centrifugation (1400 rpm, 2 minutes) the supernatant was discarded and the pellets resuspended in the new solution. At the end of the last incubation the pellets were re-suspended with calcium free HBSS and mechanically dissociated with F12 medium-coated glass Pasteur pipettes with decreasing tip diameters (tip diameters 500 μ m then 200 μ m). Neurons and accessory cells were then separated from unwanted digested tissue using a density gradient centrifugation: 1ml of cell suspension in calcium free HBSS was carefully layered on top of 4ml Percoll© /L15 medium (1:4 v/v) mixture in a 1cm wide test tube and centrifuged at 1400 rpm (380g) for 9 minutes. The pellet was resuspended with 2ml L15 medium to wash off the Percoll© and centrifuged at 2300 rpm for 3 minutes to collect the cells. The supernatant was discarded and the cells were re-suspended with complete F12 medium and were plated onto poly-d-lysine/Laminin coated glass bottom fluorodishes using culture cylinders with a 20 μ m wide inner chamber. The culture dishes were incubated at 37°C for 90 minutes for the cells to

adhere to the substrate after which the culture cylinders were removed and complete F12 medium was added.

2.3.3 Single cell PCR for jugular and nodose ganglia gene expression

Individual airway-specific jugular and nodose ganglia neurons were isolated as in the previous section, the gene expression of specific targets of interest in the relevant cell bodies was analysed using PCR based on custom primers.

2.3.3.1 Single Cell Harvesting

On the day of experimentation, animals were sacrificed with sodium pentobarbitone (200mg/kg, intraperitoneally). Nodose and jugular ganglia were dissected free of adhering connective tissue, and neurons were isolated by enzymatic digestion (*see section 2.3.2*), re-suspended in 200µL of complete F12 medium, and kept at 4°C temperature in sterile 0.5mL tubes until use.

A fluorodish was set up on the stage of a BioRad MicroRadiance 2000 Confocal Laser Scanning Microscope on a metal anti-vibration table with magnetic clamp stand holding 3-way hydraulic micromanipulator (MHW-3 Narishige, Tokyo, Japan) coupled to a 3-way micromanipulator arm (MM3 Narishige, Tokyo, Japan). A small sample of resuspended neurons (approx. 20µL at a time) were placed in the fluorodish pre-filled with 2mL of filtered (0.22µm) extracellular solution (ECS). A custom-made glass micropipette (tip ID, 70-100µm; OD, 2 mm; FIVEphoton Biochemicals, San Diego, US) set in a holder on the manipulator arm was connected to a 250 µL Hamilton glass syringe (Sigma Aldrich, UK) to generate negative pressure. Neurons were observed by brightfield, and airway neurons identified by the presence of Dil fluorescence in individual neurons. The glass micropipette was brought under the surface of the ECS while maintaining a positive pressure inside the micropipette to avoid contamination of the inside of the micropipette by any detritus in the cell media. The tip of the pipette was brought into focus and subsequently continuously observed at all stages to ensure no undesired objects entered or adhered to the tip of the pipette. Any contaminated pipettes were immediately discarded. The tip of the pipette was carefully positioned near an airway neuron using the micromanipulator. The selected neuron was carefully drawn into the tip of the micropipette using negative pressure on the Hamilton syringe. The micropipette was taken out of the medium, dismounted from its holder and its tip was gently broken into a 0.2mL micro-reaction tube containing 1µL of RNaseOUT (Life Technologies, Grand Island, NY) and placed on dry ice.

2.3.3.2 DNA digestion and cDNA synthesis

For DNA digestion and cDNA synthesis, Superscript III Cells-to-CT kit from Invitrogen was used according to the manufacturer's instructions. Resuspension buffer was added to each microreaction

tube with an isolated neuron, which was then incubated for 10 minutes at 75°C to lyse the cell. Tubes containing the neurons were then placed on ice and 6.6µL DNase mastermix (5µL DNase I, 1.6µL 10x DNase I buffer) was added to each tube. After incubating at room temperature for 5 minutes, 1.2µL of 25mM EDTA was added. The neurons were then incubated for 5 minutes at 70°C to degrade genomic DNA.

For cDNA synthesis, 3µL Oligo mastermix (1µL Oligo (dT)20, 1µL Random Hexamers, 1µL dNTP mix) was added to each tube and incubated for 5 minutes at 70°C. At this point 3.5µL of sample was added to a parallel set of tubes for –RT controls. Then 7.5µL of +RT MM (containing 6µL 5x RT buffer, 1µL RNaseOUT, 1µL 0.1M DTT, 0.83µL Superscript III) was added to the original sample tubes, and a separate -RT mastermix (with sterile water substituted for Superscript III reverse transcriptase) added to the parallel -RT tubes. The tubes were then vortexed and incubated for 50 minutes at 50°C, with a subsequent 5 minutes at 85°C step to inactivate the enzyme. The tubes were then cooled to 4°C and stored at -20°C prior to PCR amplification.

2.3.3.3 PCR amplification of targets

Primers for PCR amplification were custom-made to order (Invitrogen, UK). Where possible, previously published sequences were used, however where no primer sequences could be found, primers were designed using the primer-BLAST tool from NCBI. The most up-to-date annotation of mRNA sequences were used for each target, and primers generating approx. 20bp sequences in the range 100-300bp with a melting-point of approx. 60°C were selected. The sequences were compared within the primer-BLAST tool to the annotated transcriptome of the guinea pig, in order to check that the primers were specific for the sequence of interest. Where the intron structure of the corresponding gene was known, primers were directed against the exon-exon junction of mRNA sequences in order to minimise the risk of any potential amplification of genomic DNA.

β-actin, a gene found in all eukaryotic cells, was used as a positive control to identify if cells were successfully harvested. The parallel -RT controls were also subject to PCR amplification using the β-actin primers were also used to evaluate whether the minus RT control for each sample showed amplification. If positive, then the DNA digestion was not effective, and the corresponding +RT sample was discarded. In preliminary experiments, bath controls were taken to ensure that no contamination occurred during the process of harvesting individual neurons. No product was detected for any of the bath controls.

Protein Gene Product 9.5 (PGP9.5) is encoded by the gene ubiquitin C-terminal hydrolase 1 (UCHL-1) and is highly expressed and specific to neurons. Expression of UCHL-1 was determined in all samples to confirm that a neuron had been harvested.

Primers were used with the custom primers to run parallel PCR reactions on each harvested neuron using HotStarTaq DNA Polymerase kit (Qiagen, UK) according to manufacturer's instructions. 1 µL sample was mixed with 19µL Master Mix (containing 0.5µL forward primer (10µM), 0.5µL reverse primer (10µM), 0.1 µL HotStarTaq, 2µL 10x buffer, 0.8µL MgCl₂, 0.5µL dNTP, 0.5µL Water). Prepared reaction mixes were cycled on a PCR machine (StepOnePlus qPCR or Veriti™ Thermal Cycler, Applied Biosystems, UK) with parameters as described in Table 2.1.

1 cycle	95°C	15 min
<u>50</u> cycles	95°C	30 sec
	60 °C	30 sec
	72°C	1 min
1 cycle	72°C	10 min
	4°C	1 min

Table 2.1 PCR program

2.3.3.4 Visualisation of PCR products

After PCR amplification was completed, samples containing loading buffer were loaded into a 3% (v/w) agarose gel containing 5µl/100ml Safeview for UV visualisation of cDNA products. The gel was placed into an electrophoresis tank (PowerPac 200, BioRad, US) filled with TBE buffer and 150V was applied for approx. 1h. Bands of product were then visualised under UV light. Where bands were visible a neuron was considered to express the target, where no band was visible a neuron was considered not to express a target. cDNA from a tissue known to express the target was run in each PCR reaction and in each gel as positive as positive controls in order to confirm the PCR reaction and electrophoresis techniques were successful.

2.3.4 Intracellular calcium imaging on isolated vagal ganglia neurons

Airway sensory neurons were harvested and primary cultured following the protocol described in chapter 2.3.2. Neurons were seeded in fluorodishes in 2ml complete F12 medium. The neurons were left to adhere securely overnight in a culture chamber at 37°C with 5% CO₂ and used for calcium imaging experiments the next day¹²⁵.

2.3.4.1 Fluorescence imaging dyes

The membrane permeable, Ca^{2+} ratiometric dye Fura2-AM was used to measure intracellular levels of calcium ($[\text{Ca}^{2+}]_i$) in vagal ganglia neurons. Acetoxymethyl (AM) ester group bound to the Fura2 molecule provides its membrane permeable property. Fura2-AM was dissolved in DMSO and then mixed with equal volume of Powerload TM (From ThermoFisher Scientific; water soluble F-127 pluronic[®] acid with pH compensated to 7.4) to increase solubility. The mixture was then diluted 1:100 with extra cellular solution (ECS) medium to obtain a loading solution containing 12 μM of Fura2-AM.

Ganglia cells were incubated with the loading solution for 40 minutes in the dark at room temperature. The fluorodishes were then washed twice with ECS and kept in the dark for at least 30 minutes at room temperature to allow for the intracellular diesterases to remove the AM group associated to the Fura2 molecule trapping it in the cytosol. The fluorodishes were then rinsed twice with room temperature ECS and twice with 37°C ECS, placed on the microscope stage at 37°C and let to rest in the dark for 15 minutes before starting experiments.

The peak excitation and emission wavelength of Fura-2 in presence and in absence of calcium are as listed below alongside the peak excitation and emission wavelengths of the retrograde tracer used to identify the airways innervating neurons (Table 2.1).

Dye	peak excitation wavelength	peak emission wavelength		
			Low calcium	High calcium
Fura-2	$\lambda = 380 \text{ nm}$ 	$\lambda = 512 \text{ nm}$ 	High intensity	Low intensity
	$\lambda = 340 \text{ nm}$  (U.V.)	$\lambda = 510 \text{ nm}$ 	Low intensity	High intensity
DilC18	$\lambda = 555 \text{ nm}$ 	$\lambda = 570 \text{ nm}$ 		

Table 2.2 Dyes and its peak excitation/emission wavelength

Images were acquired using an inverted epifluorescence microscope (Zeiss) equipped with a mercury arc lamp. The dishes were placed onto a microscope stage housed in a 37°C incubating chamber. An Hamamatsu cooled CCD camera and HCLImage software (Hamamatsu) was used for image acquisition. The microscope was equipped with an excitation and emission filters set and a set of dichromatic mirrors appropriate for the fluorescent dyes employed (Carl Zeiss Microscopy):

- Excitation filters 335/7 nm and 387/11 nm, Beam Splitter 410 nm, and Emission filter 510/10 nm for Fura2.

- Excitation filter 531/40 nm, Beam Splitter 565nm, and Emission filter 593/40 nm for DiIC18.

Figure 2.4 shows a schematic and a photo of the epifluorescence microscope used.

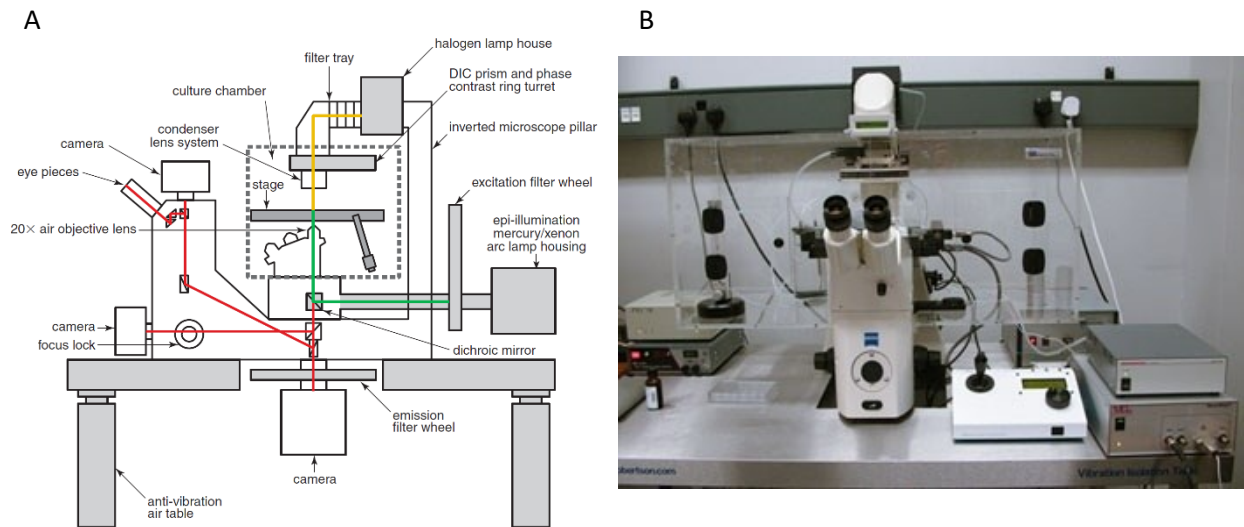


Figure 2.4 Epifluorescence microscope setup

A: Schematic of the epifluorescence microscope; B: A picture of the microscope used.

All solutions were kept at 37°C and applied with a perfusion system allowing a rapid change of the dish content (Figure 2.5). Perfusion tubes were placed with their tips on opposite side of the dish. The extraction tip should reach the bottom of the dish to allow a complete extraction except for the inner chamber of the fluorodish (300µl) protecting the cells from being in contact with air while changing the solutions. Drugs containing solutions were applied by bolus of 2.5ml.

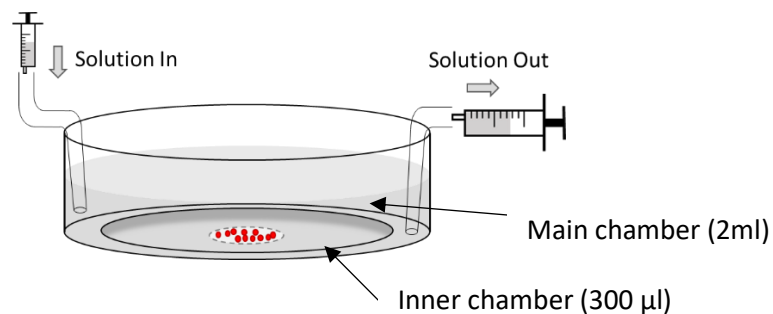


Figure 2.5 Design of solution-changing perfusion system

The chamber is comprised of a main chamber (2ml volume) on top of an inner chamber (300 µl volume) where the cells are located. When the system's chamber is perfused the upper chamber is emptied and filled and the cells in the inner chamber are at all-time protected by a layer of medium.

2.3.4.2 Intracellular calcium level recording

Airway-innervating neurons were identified by the presence of Dil fluorescence (Figure 2.6). In the beginning and end of each experiment, 50mM potassium chloride (K50) was applied for 30 seconds and the response was used to normalise further responses and test cells viability. After K50 was washed out, vehicles and drug applications were time matched. Each stimulus application was followed by a washout with ECS for a minimum of 10 minutes or longer if needed until the intracellular calcium levels returned to resting state. During key parts of the experiment while stimulus and stimulus plus vehicle/drug were applied images were recorded with a frequency of 1 frame every 10 seconds. During antagonist/vehicle and wash out images were recorded every minute to minimise photo bleaching of the dye.



Figure 2.6 Dissociated and primary cultured Jugular neurons

A: D.I.C image in Brightfield light transmission of sensory neurons (white arrows) and accessory cells (black arrows). B: Neuronal tracker (Dil) fluorescence acquired at 590nm identifying airway neurons. C: Combined image.

2.3.4.3 Data analysis

The imaging recordings were analysed using Fiji software²⁰². The light emitted at 510 nm after stimulations at 340 and 380 nm was measured over time for each neuron identified as airway innervating. The respective surrounding background was subtracted from the signals recorded for each neuron. The measured signals were uploaded into a Microsoft Excel macro spreadsheet to calculate the fluorescence ratio of the signal emitted at 510nm when excited at 340nm divided by the signal emitted at 510nm when excited at 380nm. The use of the ratiometric measurement permit for a stable signal and free us from artefacts due to degradation of the dye by photo-bleaching and inter-experimental differences in loading of Fura2-AM. The baseline of the ratio was subtracted and the responses to drugs application were calculated using the area under curve (A.U.C) of the response. AUC allows us to take into account the overall intensity of calcium elevation in the cytosol overtime. Data were normalised as a percentage of the initial A.U.C. K50 response and expressed as mean $\pm$ SEM.

2.3.5 Oxidative imaging on isolated vagal ganglia neurons

Airway sensory neurons were harvested and primary cultured following the protocol described in chapter 2.3.2. Neurons were seeded in fluorodishes in 2ml complete F12 medium. The neurons were left to adhere securely overnight in a culture chamber at 37°C with 5% CO₂ and used for oxidative imaging experiments the next day¹²⁵.

2.3.5.1 Superoxide sensitive fluorescent dye

Superoxide sensitive fluorescent dye di-hydro-ethidium (DHE) was used to measure oxidative responses to stimuli in isolated vagal neurons.

The peak emission wavelengths of DHE are 480 nm (light blue) and 600 nm (bright orange-red) when respectively exposed to peak excitation wavelengths of 365 nm (near UV blue) and 515 nm (light green). Signals were recorded using the same imaging system as the one used in calcium imaging experiments (as in paragraph 2.3.3). In the cytosol, DHE exhibits light blue fluorescence (480 nm) in response to excitation with near UV light (365 nm) (Figure 2.13). When it is oxidised, it moves into the nucleus and intercalates within the cell's DNA emitting a bright orange-red fluorescence (600 nm) upon excitation with green light (515 nm). A mercury arc lamp was used with 2 sets of filters:

- Excitation filters 365/30 nm (deep blue), Beam Splitter 400 nm, and Emission filter 485/20 nm (light blue) for non-oxidised DHE.
- Excitation filter 520/21 nm (light green), Beam Splitter 550 nm, and Emission filter 593/40 nm (bright orange-red) for oxidised DHE.

The shift on excitation and emission wavelengths upon oxidation allowed to record a pseudo-ratiometric signal.

Dye	peak excitation wavelength	peak emission wavelength
DHE non oxidized	$\lambda = 365$ nm 	$\lambda = 480$ nm 
DHE oxidized	$\lambda = 515$ nm 	$\lambda = 600$ nm 

Table 2.3 Di-hydro-ethidium dye and its peak excitation/emission wavelength

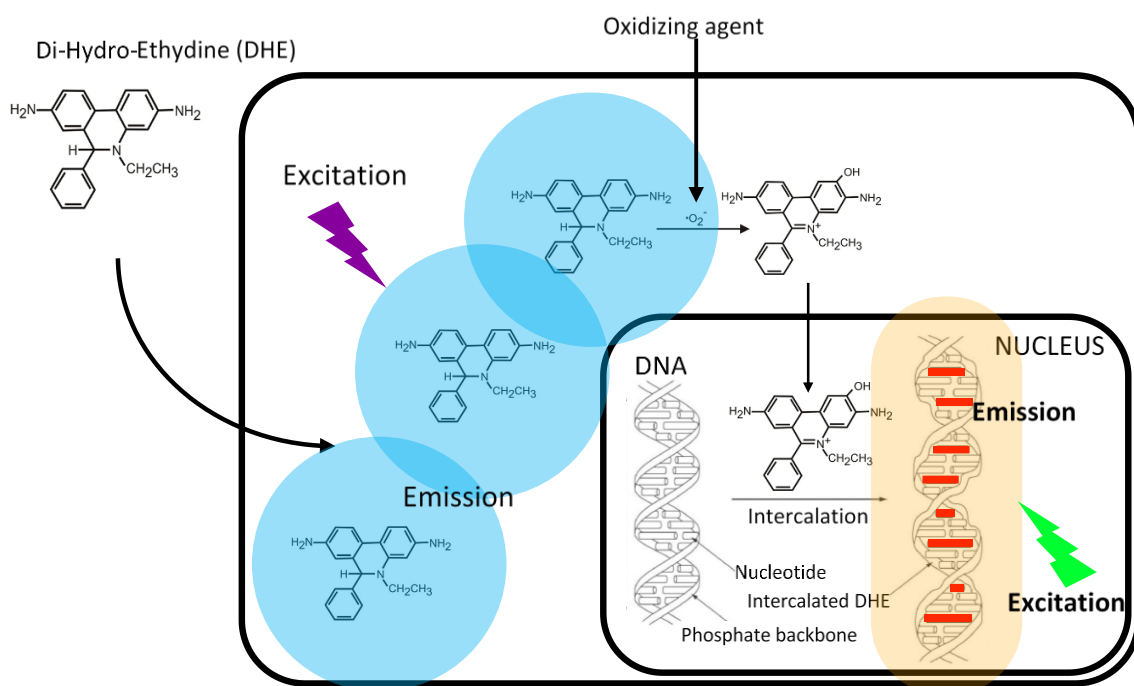


Figure 2.7 Schematic of the mode of action of di-hydro-ethidium

Ganglia neurons were washed with ECS three times to remove the culture medium and then loaded with DHE at $6\mu\text{M}$ in ECS for 30 minutes at 37°C in the dark. Prior to imaging, neurons were washed three times with ECS at a 5-minute interval to remove all DHE from the solution. All solutions (kept at 37°C) were injected and extracted with the same perfusion system used in calcium imaging experiments.

2.3.5.2 Superoxide signal recording

The measurement of oxidative stress was characterised using increasing concentrations of H_2O_2 ($0.1\mu\text{M}$, $1\mu\text{M}$, $10\mu\text{M}$) which is reported to increase cellular oxidative reactions as standard.

During the experiments, the initial stimulus was applied for 3 minutes. Then inhibitors or vehicle were incubated for 10min and stimulus plus vehicle/drug were applied for the same length of time as the initial stimulus. After washout a final stimulus was applied again for 3minutes. Images were recorded at a frequency of 0.1 Hertz (1 image every 10 sec). Before each injection, 10 frames were recorded as baseline. After each stimulus application, neurons were carefully washed with ECS six times.

2.3.5.3 Data analysis

The recordings were analysed using Fiji software²⁰². For each neuron, a region of interest were drawn around the neuron to the exclusion of the nucleus to measure the light emitted at 480 nm (non-oxidized DHE). Simultaneously a region of interest was drawn around the nucleus to measure the light

emitted at 600nm (oxidized DHE). (Figure 2.14) Once oxidized the DHE excitation and emission fluorescence undergoes a large wavelength shift. Since oxidized DHE bound to DNA is very stable and respond only to 515nm light but do not emit fluorescence upon excitation to 365 nm whereas cytosolic non-oxidized DHE do not emit light upon excitation to 515 nm but only after excitation with 365nm, both emission signals were used to calculate a pseudo ratiometric signal where the light emitted after excitation with 515nm was divided by the light emitted after excitation with 365 nm: F_{515}/F_{365} . Background fluorescence around the cell for each emission wavelengths was recorded and respectively subtracted from the signals.

Once the pseudo ratio was obtained, the baseline before each event was subtracted (Figure 2.14 E) and the difference of ratio prior and after addition of the oxidizing agent was measured. Each increase in ratio shows an oxidizing event taking place in the cell due to superoxide. The generation of superoxide was measured as the peak of the signal minus the baseline recorded prior to adding the drug (Figure 2.14 D, E) and data were expressed as mean $\pm$ SEM.

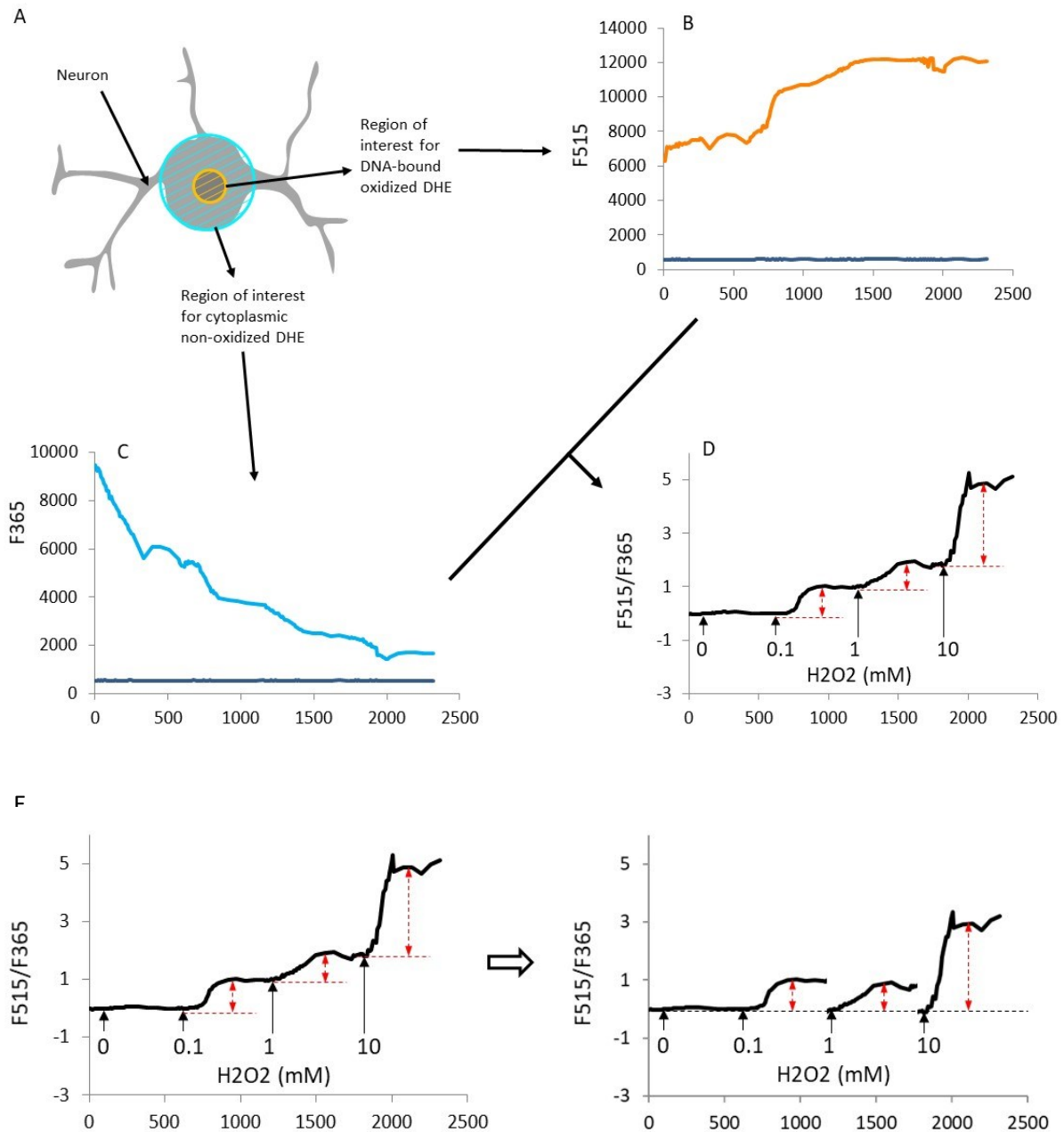


Figure 2.8 Schematic of the analysis of DHE signal

A: region of interest to measure the non-oxidized DHE signal (blue, cytosol) and the oxidized DHE signal (yellow, nucleus). B: Signal obtained from the nucleus overtime (yellow trace) and background fluorescence for this wavelength (dark blue line). C: Signal obtained from the cytosol overtime (blue trace) and background fluorescence for this wavelength (dark blue line). D: pseudo ratio ($F515/F365$) obtained after subtracted background fluorescence, dividing the nucleus signal ($F515$) by the cytosol signal ($F365$) and subtracting the initial baseline. E: Ratio $F515/F365$ with the baseline each signal obtained after addition of oxidizing agent subtracted.

2.4 Isolated vagus nerve depolarisation recordings

Afferent vagal nerve fibres conduct stimuli detected in the airways to the brain, where the input signal can trigger respiratory reflexes such as cough or bronchospasm. This technique measures the depolarisation of the whole nerve, the step prior to initiation of action potential, caused by a change in the membrane potential of a large amount of nerve fibres in the nerve trunk. It is a semi high-throughput technique that can test a wide range of compounds that could potentially cause or inhibit cough. Animal tissues (guinea pig and rats) were primarily used for investigations, and key experiments were done on human vagus from donors to assess the translation of the findings in animal models. This is a whole-tissue technique as opposed to section 2.3 looking at individual responses. In this technique it is not possible to differentiate airway from non-airway sensory nerve responses, and the airway terminals are not present. However key advantages are that the structure of the neuron is retained, this technique is higher throughput/less labour intensive, and human tissue can be used.

2.4.1 Vagal nerve dissection

Vagal nerves from rats, GPs and human donor tissue were used in this system. Guinea pigs or rats were sacrificed with sodium pentobarbitone (200mg/kg, i.p.). The neck was opened by midline incision. The ribcage was removed and then the vagus nerves on both sides of the trachea were cleared of most of the surrounding muscles and viscera. The vagus nerves were then carefully dissected and placed in modified Krebs-Henseleit solution ("Krebs" containing in mM: NaCl 118; KCl 5.9; MgSO₄ 1.2; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25.5; glucose 5.6) and bubbled with 95% O₂ / 5% CO₂. After dissecting the nerve free from connective tissue, each vagus nerve was carefully desheathed. Vagus nerves were then cut into segments approximately 10-15mm long, and a piece of string tied to one end to aid mounting in the recording chambers. Human vagus nerves parallel to the trachea were similarly dissected except from en bloc lung tissues received as described in section 2.2.3. Prior to experimentation, all nerve segments were kept in oxygenated Krebs solution at room temperature.



Figure 2.9 Image of a section of human vagus taken during dissection of the nerve

2.4.2 Recording of vagal nerve depolarisation

A 'grease-gap' recording chamber (Figure 2.4) was used to record nerve depolarisation as previously described⁸³. The nerve was connected to a string and drawn through a narrow channel (2mm diameter) in a Perspex block. Care was taken to make sure the nerve was not stretched or damaged in any way. 2mm diameter glass rods connected to Ag/AgCl pellet half-cell electrodes (Mere 2 flexible electrodes, WPI, UK) were carefully filled with Krebs solution and placed onto both ends of the nerve. Vaseline™ petroleum jelly was then injected into a port around the centre of the nerve segment, in order to electrically and chemically isolate the two ends of the nerve from each other.

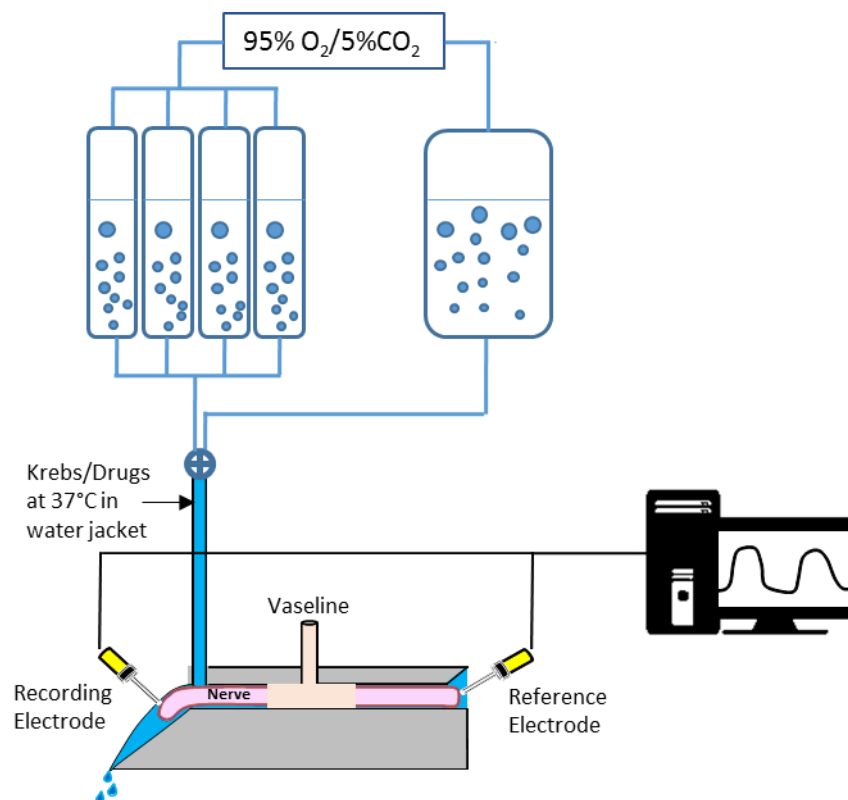


Figure 2.10 Apparatus for recording isolated vagus nerve depolarisation

One end of the nerve connected to the recording electrode was perfused with either Krebs or Drugs at a rate of 2mL/min. The other end of the nerve in a chamber containing Krebs solution was connected to a reference electrode. When nerve activators were perfused onto the nerve ending next to the recording electrode, Na⁺ ions from the perfused Krebs to move into the cell (i.e. action potential generation), leaving excess Cl⁻ ions interacting with the AgCl pellet of the recording electrode. By contrast, when the stimulus was removed by perfusion with Krebs, K⁺ ions leave the nerve (i.e. repolarisation), reducing the association of Cl⁻ ions with the AgCl pellet of the recording electrode. The use of Vaseline to isolate the end of the nerve next to the reference electrode leads to the generation of an electrical potential difference between the two ends of the nerve. In order to record this

potential difference, the recording and reference electrodes were connected to a DAM50 differential amplifier unit (WPI, UK) which amplified the DC potential signal (x10 gain, high filter 1KHz, 5Hz sample rate). The amplifier signal was recorded using a LabTrax 4-Channel Data Acquisition unit (WPI, UK) connected to a computer running LabScribe data acquisition software (v3.5, iWorx, UK) running on Windows 10 (Microsoft, US). A 1mV pulse from the amplifiers was used to calibrate the data acquisition device at the start of each experimental day. Data was acquired at a rate of 200Hz, and the raw signal was monitored for excessive noise, whilst a real-time computed signal (Savitsky-Golay smooth of 221 rolling points) was monitored and later analysed to observe and measure signal amplitude in response to chemical stimulation of the nerve. Krebs solution and agonists or antagonists dissolved in Krebs were kept in syringes above the chambers, connected to manifold stopcocks to allow changing of the perfusate flow between the Krebs solution reservoir and the various drugs. All solutions were bubbled with 95% O₂ / 5% CO₂ and maintained at 37°C by heated water jackets.

The general protocol for experiments to determine the ability of a stimulus to cause nerve activation was as follows. Non-cumulative concentration responses to potential stimuli were assessed. The tissue was mounted in the chamber and allowed at least 10 minutes to stabilise whilst being perfused with Krebs alone. Then the nerve was perfused with a single concentration of a stimulus for two minutes, and then Krebs until the signal returned to baseline, and this repeated with the full range of concentrations. A similar stimulation with vehicle was assessed to ensure no effect.

The general protocol for experiments to determine the inhibitory activity of a test compound was as follows. The tissue was mounted in the chamber and allowed at least 10 minutes to stabilise whilst being perfused with Krebs alone. Then the nerve was perfused with a stimulus for two minutes, and then Krebs until the signal returned to baseline. Up to two subsequent stimulus responses were then performed to assess the initial viability of the tissue, and achieve two reproducible internal control responses. By the end of each stimuli challenge, the nerve was washed with Krebs until baseline is re-established. The nerve was then treated with vehicle or test compound for 10 minutes, immediately followed by a 2-minute stimulation with same stimulus in the presence of the test compound. Following a wash out period, the tissue was perfused again with the stimulus for 2 minutes to confirm the tissue was still viable (Figure 2.5).

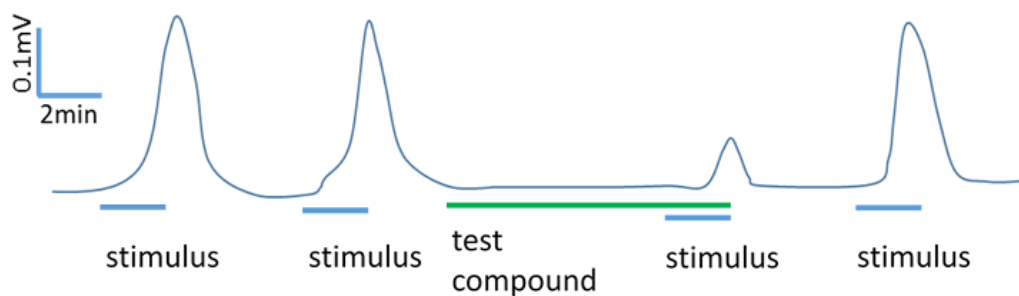


Figure 2.11 Example illustration of vagus nerve depolarisation recording using protocol to measure inhibitory activity of test compound

2.4.3 Data analysis

The magnitude change in voltage (mV) from baseline was calculated for each stimulus.

For concentration responses to potential stimuli, magnitude change in voltage (mV) from baseline was calculated for each repeated experiment performed on a piece of tissue from an individual animal, and data calculated and presented as mean $\pm$ S.E.M. Following characterisation, a sub maximal concentration of the stimulus was taken forward for antagonist experiments.

If antagonists were involved in the experiments, percentage inhibition was calculated as follows. Initially the internal control responses were averaged to give a single value. Then the following calculation was applied:

$$(100 - (\text{stimulus in presence of test compound}) \div (\text{average internal control response})) \times 100$$

Percentage inhibition was calculated for each repeated experiment performed on a piece of tissue from an individual animal, and data calculated and presented as mean $\pm$ S.E.M.

2.5 *In vivo* single fibre action potential firing recordings

This technique assesses the effect of test compounds on action potential firing of single afferent airway terminating fibres. It involves an *in vivo* anaesthetised guinea pig single fibre preparation with both vagal nerves cut at the central end. Therefore the reflex arc was stopped and any responses were effects on the nerve fibre itself. Guinea pig and rats were used for investigations. This technique has the advantage of the compounds acting on the nerve termini, where action potentials would normally

be evoked by stimuli, and measuring nerve firing as an end-point, compared to the isolated vagus nerve (section 2.4) described previously.

2.5.1 Surgery and recording set up

Male guinea pigs or male Brown Norway rats were anaesthetised with urethane (i.p. 1.5 g/kg), and supplemented with additional urethane as required.

The trachea was cannulated with a short length of Perspex tubing and blood oxygen and pH maintained by ventilation (Ugo Basile small animal ventilator), with a tidal volume of 10 ml/kg and 50-60 breaths/min of laboratory air. The right jugular vein was cannulated for injection of drugs and the right carotid artery was cannulated, passing to the ascending aorta/aortic arch, to measure systemic arterial blood pressure using a pressure transducer. Heart rate was measured from the arterial pulse trigger. Arterial blood pressure and heart rate were constantly recorded on the Spike 2 software data acquisition system via a CED Micro1401 interface. Trachea pressure was measured with a pressure transducer connected to a side arm of the tracheal cannula and recorded with the Spike 2 software data acquisition system via a CED Micro1401 interface. Body temperature was continuously monitored by a rectal thermometer and 37°C was maintained with a heated blanket and control unit (Harvard).

Guinea pigs were paralysed with vecuronium bromide (0.1 mg/kg i.v.), followed every 20 min with 0.05 mg/kg i.v. for maintenance. Rats were paralysed with pancuronium bromide (0.1 mg/kg i.v.), followed every 20 min with 0.05 mg/kg i.v. for maintenance. The depth of anaesthesia was frequently assessed by monitoring the response of heart rate and blood pressure to noxious stimuli.

To locate the cervical vagus nerves, a cervical incision was made. The cervical vagus nerves were dissected from the carotid artery, sympathetic and aortic nerves and cut at the central end. The left vagus nerve was cleaned for sensory nerve fibre recording. The skin and muscle in the neck at either side of the incision were lifted and tied to a metal ring to form a well. The well was filled with light mineral oil to stop electrical signals interacting with each other. Bipolar Teflon-coated platinum electrodes were used for recording, using fascia positioned on one electrode for a reference.

The vagus nerve was placed on a small black perspex plate for subsequent dissection. Under a binocular microscope, thin filaments of nerve were teased from the vagus nerve and placed on the second electrode until a single active unit, or one of not more than two or three units, was obtained. Action potentials were recorded using electrodes connected to a pre-amp headstage (Digitimer NL100K). The signal was amplified (Digitimer NL104), filtered (Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) and then input into the Micro1401 interface and the

Spike 2 software. The software can allow pulse train counting over selected time periods. In addition, the input signal to the Spike software was monitored on a digital storage oscilloscope (Tektronix DPO 2012). The input signal was also transmitted to a loudspeaker (figure 2.15).

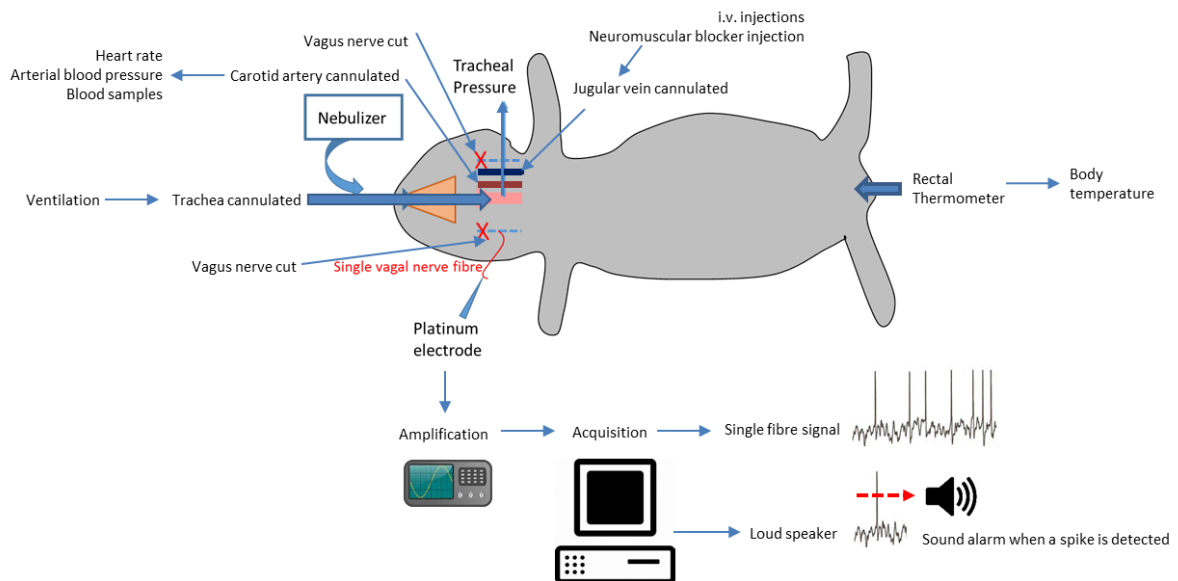


Figure 2.12 Apparatus setup for in vivo single afferent fibre recording

2.5.2 Nerve fibre identification

Single vagal nerve fibres are classified as originating from the major groups of airway sensory nerve endings. Several criteria⁷⁸ can be used to differentiate slowly adapting stretch receptors (SARs), rapidly adapting stretch receptors, (RARs, irritant receptors, A δ -fibres), and pulmonary/bronchial C-fibre receptors. All single fibre firing recording data in this thesis has been carried out on C-fibres. A receptor that has no obvious pattern to the spontaneous activity, no response to hyperinflation/hyper deflation, but response to capsaicin aerosol will be identified as a C-fibre.

At the end of each experiment conduction velocities were measured to distinguish slow conducting non-myelinated C-fibres from fast conducting myelinated A-fibres. Conduction velocity was calculated by recording time lag between stimulation and recorded action potential to the distance between stimulating and recording electrodes. The vagus nerve was stimulated close to the thorax with bipolar silver electrodes, using a supra threshold voltage at 0.5ms, 1 Hz (Grass stimulator). The corresponding action potential was recorded in the nerve fibre under observation and was acquired on the Spike software screen in a single sweep.

2.5.3 Measurement

After surgery, the animals were allowed to stabilise for at least 30 min. Following identification of an airway afferent fibre, the fibre was investigated by a more detailed protocol in each chapter.

An aerogen nebulizer (Buxco Nebulizer Controller - 5) was spreading drugs and was connected to the ventilator so that inspired air was passed through the medication chamber before entering the lungs of anaesthetized animals via the tracheal cannula. Finally, all the data were recorded on the Spike 2 data acquisition system and stored for subsequent analysis. All animals were sacrificed at the end of the experiments with an overdose of pentobarbitone.

2.6.4 Data Recording and analysis

The results are expressed graphically (Mean $\pm$ S.E.M.) as bar charts and data were analysed as follows:

Total impulses were counted throughout the course of the experiment in 1 min bins and the data expressed as total imp s⁻¹ and plotted as bar charts. Paired t-tests (GraphPad Prism software) were used to compare before and after treatment. Values of P<0.05 were considered significant.

2.6 *In vivo* guinea pig cough recordings

This technique was utilised to investigate the inhibitory effect of compounds on the cough response elicited by a challenge agent. Guinea pigs were used for this technique because unlike rats and mice, guinea pigs have a cough response similar to humans¹²⁷.

2.6.1 Cough challenge apparatus and setup

For cough recordings, conscious unrestrained male Dunkin Hartley guinea pigs were placed into transparent plethysmography chambers (Buxco, Wilmington USA). The chambers were attached to a central Aerogen nebuliser (Buxco, US), which allowed simultaneous aerosolisation of compound and visualisation of 2 guinea pigs at one time. Both chambers were equipped with a microphone attached to an amplifier and a speaker and cough was detected as previously described²⁰³. Briefly, guinea pigs were allowed 2-3 minutes to acclimatise, and then a 2 minute baseline was recorded before the aerosolised tussive challenge was administered, and cough was recorded. The tussive stimulus was aerosolised for 10 minutes and coughs manually counted by two trained observers blinded to treatment. The means of the 2 counts were used. Guinea pigs would be monitored for any adverse health effects during this time period, and at the end of the experiment euthanised with an overdose of pentobarbitone (200mg/kg i.p.).

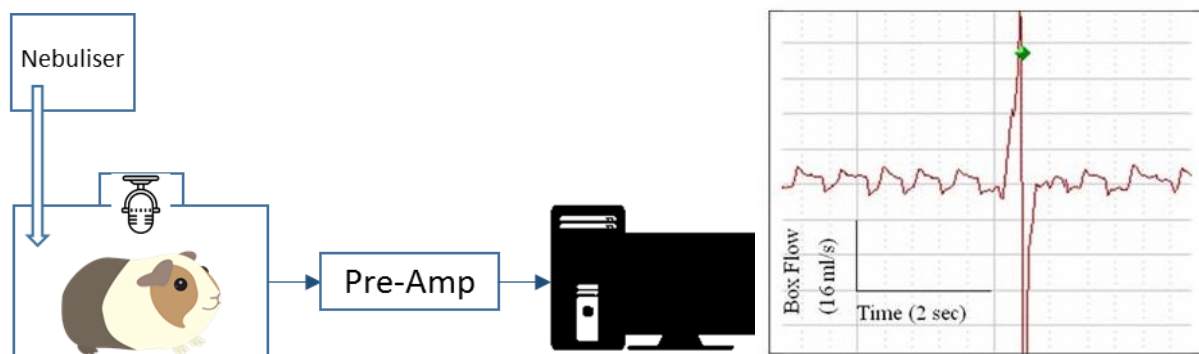


Figure 2.13 Cough recording device and example of a cough trace recorded in guinea pigs

2.6.2 Data recording and analysis

Cough and Penh were recorded for the 10 minute period including five minutes of aerosol challenge. Coughs were counted manually by two trained observers, blinded to treatment groups. Coughs were detected by the characteristic change in posture of the animal along with pressure changes and sound (Figure 2.16).

2.7 *In vivo* OVA driven rat model of the Late Asthmatic Response

The Ovalbumin (OVA) driven rat model of the Late Asthmatic Response (LAR) has been previously characterised in the group²⁷. This is a model that mimics the allergic inflammatory response in asthmatics by using ovalbumin (OVA) protein to sensitise and challenge the animals. Following previous in house data, two control groups were used; OVA-Sensitised/Saline-Challenged and OVA-Sensitised/OVA-Challenged. Throughout the studies, 20mg/ml aluminium hydroxide and 20mg/ml magnesium (AlumTM) diluted 1:1 with saline was used as an adjuvant during the sensitisation of animals.

2.7.1 Sensitisation and Challenge

Male Brown Norway rats (200-225g) were sensitised with OVA in AlumTM (100µg per rat, 1ml i.p.) on days 0, 14 and 21 (see figure 2.1). On day 28, rats were placed in a Perspex chamber and challenged with 1% (w/v) aerosolised OVA for 30minutes. Compounds that may affect or alter the LAR can also be administered both before and after challenge and are detailed in relevant chapters.

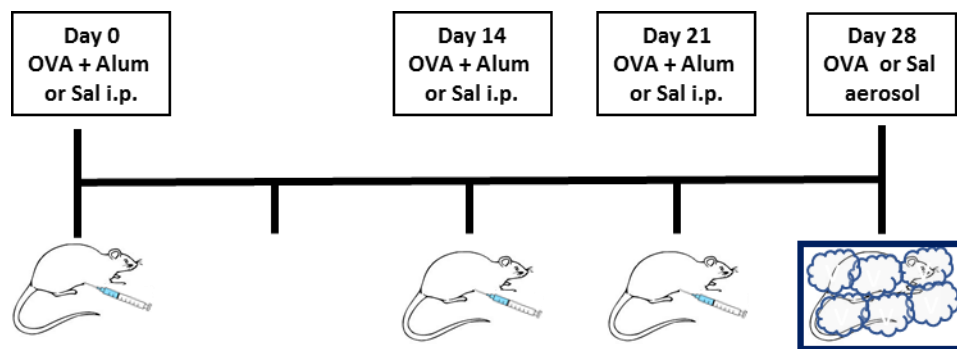


Figure 2.14 Timeline of OVA-sensitised rat model of LAR

2.7.2 Assessment of lung function

The LAR is a key feature of asthma. It is a parasympathetic reflex following allergen challenge which causes prolonged bronchospasm peaking at 2-3 hours and lasting up to 6 hours. In the studies described, bronchospasm was monitored using Flow Whole Body Plethysmography (FWBP) and expressed as enhanced pause (Penh), which has been used as an indicator of lung function and arbitrary measure of constriction throughout this thesis. This is a non-invasive method where the animals are conscious and unrestrained and therefore have more natural breathing patterns.

FWBP measures pressure changes between the main chamber and reference chamber by encompassing a pneumotachograph in its wall. Within this technique, a respiratory waveform is recorded which is a result of pressure changes within the chamber which occur during respiration. During inspiration, the chamber pressure decreases as air is drawn from the chamber into the animal, but this then results in thoracic displacement where the pressure change increases. As the air moves from the chamber condition to body conditions it is conditioned (heated and humidified) and this results in a net increase in pressure during inspiration. As nasal flow lags behind thoracic flow, there is a delay before air is driven into the lungs due to resistance, which is greater during times of bronchoconstriction. The difference between the two flows make up the FWBP waveform, and 'enhanced pause' (Penh) is calculated from this waveform.

Following OVA aerosol, rats were placed in rat WBP chambers (Buxco Electronics, USA). Pressure changes were recorded from 30minutes following the end of OVA challenge using Buxco Finepointe Software (Buxco Electronics, USA). Average Penh was recorded at 10minute intervals for a total duration of 5 hours and was used for analysis²⁰⁴.

2.7.3 Data Analysis

The Penh average was plotted against time following challenge. The Area Under Curve for the various experimental groups was calculated and used for statistical analyses as detailed in the relevant chapters.

2.8 Data Statistical Analysis

I summarised types of data analysed with each statistical test used in this thesis below:

- For data normalised to an internal control (i.e. isolated vagus nerve, neuron and in vivo single fibre experiments), statistical test was performed on the raw data rather than the normalised data.

In these cases, I used paired student's t-test analysis, comparing the response in the presence of vehicle or test compound to the initial control response. For multiple groups dosed with different concentrations, I did not examine whether a concentration dependent effect exists between different concentrations, as the study is not powered for that. For the same reason, I also tested single concentrations on each piece of nerve, instead of cumulative concentration response curves. Observational instead of objective analysis is used to determine whether there is a concentration dependent effect.

- For data where made a comparison between the mean responses in two separate groups of animals (i.e. LAR and cough studies), I used a Mann–Whitney U test to compare the responses in vehicle-treated group to compound-treated group.
- When the comparison was between the mean responses of more than two separate groups of animals (i.e. LAR studies with more than one dose/drug being tested), I used a Kruskal-Wallis test to compare the responses in the vehicle-treated group to the responses in each of the drug-treated groups.

2.9 Pharmacological compounds

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

	Compound	Potency/Choice of concentration	Reference
TRPV1 agonist	Capsaicin	pEC ₅₀ 6.1 - 8 (mouse, rat, human)	205–207
TRPV1 antagonist	XEN-D0501	pIC ₅₀ 10.1 (human)	208,209

TRPV4 agonist	GSK1016790a	pEC ₅₀ 7.7 - 8.7 (mouse, rat, human)	210,211
TRPV4 antagonist	GSK2193874	pIC ₅₀ 7.4 - 8.7 (mouse, rat, human)	212–214
TRPV2 antagonist	Tranilast	pIC ₅₀ 5.0 (human)	215–217
	SKF96365	pIC ₅₀ 5.0 - 6.3 (human)	218,219
	NK-4	pIC ₅₀ 5.5 (human)	220
TRPA1 agonist	Acrolein	pEC ₅₀ 5.3 (human)	83,221
	AITC	pEC ₅₀ 4.5 - 4.7 (mouse, human)	140,222
	PGE ₂	PGE ₂ (10 µM) was used in the same system in the group as reported.	84
	Bradykinin	Bradykinin (3 µM) was used in the same system in the group as reported.	84
	H ₂ O ₂	pEC ₅₀ 3.5 (human)	223
	Diesel exhaust particles	Diesel exhaust particles (1 µg/mL) was used in the same system in the group as reported.	145
TRPA1 antagonist	Janssen 130	10 µM was used in the same system in the group as reported.	145
	HC-030031	HC-030031 (10 µM) was used in the same system in the group as reported.	83,84
GPR35 agonist	TC-G1001	pEC ₅₀ 5.6 – 7.1 (mouse, rat, human)	224
	Kynurenic acid	pEC ₅₀ 4.4 – 5.2 (mouse, rat, human)	225
	Zaprinast	pEC ₅₀ 6.1 (human)	226
	HTL227477	Dosed 0.003 - 0.3mg/kg into rats on internal report	Not shown
GPR35 antagonist	ML145	pIC ₅₀ 7.4 (human)	227
	CID2745687	pIC ₅₀ 6.3 – 7.2 (human)	227
NADPH oxidase (NOX) inhibitor	VAS2870	pIC ₅₀ 5.0 - 6.1 (rat, human)	228,229

Table 2.4 Pharmacological compounds

Chapter 3 Investigation into the impact of cromoglycate on a preclinical rodent model of the late asthmatic response

3.1 Rationale

Asthma is a chronic disease of the airways characterised by reversible bronchoconstriction, inflammation and symptoms such as cough and wheeze. Allergen challenge in asthmatics results in bronchoconstriction which occurs in two stages; the Early Asthmatic Response (EAR) which is immediately after challenge²³⁰ and the Late Asthmatic Response (LAR) occurring approximately 3-8 hours following allergen exposure²⁷. Within the Respiratory Pharmacology group, the LAR has been modelled successfully in Brown Norway rats, with which they have shown that the LAR, but not the EAR, is an airway sensory nerve mediated parasympathetic reflex^{27,231}. The requirement of airway sensory nerves in the model of LAR was shown through the use of anticholinergic drugs and the fact that it was abolished under anaesthesia²⁷. This suggests that the parasympathetic cholinergic reflex is driving the LAR response.

Disodium Cromoglycate (DSCG) has been utilised as an asthma therapy for a number of years as it is known to modulate symptoms with a favourable safety profile¹⁶¹. It was presumed that these effects of DSCG could be attributed to its effect as a mast cell stabiliser^{170,232} but other mast cell stabilising compounds have failed as asthma therapeutics¹⁸⁵, indicating that the beneficial effects of DSCG may be via alternative mechanisms. Furthermore, despite these promising clinical properties, the use of DSCG is limited because of its short pharmacokinetic (PK) profile and corresponding limited duration of action. Indeed patients require treatment with DSCG four times a day²³³. If one could determine DSCG's mechanism of action of DSCG, it may be possible to develop a novel therapy with a longer PK profile and thus be a more useful treatment.

TRPA1 is a member of the Transient Receptor Potential family of ion channels which are expressed on airway sensory nerves^{87,234}. Activation of TRPA1 has been demonstrated to activate airway sensory nerves and initiate reflex events such as cough in animals and man⁸³. Previous work within the Respiratory Pharmacology group has shown that activation of the airway sensory nerves and triggering the parasympathetic response in the rat model of LAR was dependant on the TRPA1 ion channel²⁷. DSCG has also been shown to attenuate the LAR in clinical and preclinical studies^{177,178}, and has also been shown to inhibit TRPA1 triggered sensory nerve activation in the gastrointestinal tract²³⁵. Therefore the hypothesis to be tested in this chapter is that DSCG attenuates LAR by inhibiting TRPA1 ion channels on airway sensory nerves.

The initial aim of this chapter was to confirm the inhibitory effects of DSCG and TRPA1 blocker in this model of LAR. Once this was established, the next aim was to determine if DSCG can attenuate TRPA1 induced activation of airway sensory nerves using a range of systems e.g. isolated vagal nerve, airway specific neurons, *in vivo* sensory nerve recordings and a cough model.

3.1.1 Chapter hypothesis

DSCG attenuates LAR by reducing TRPA1 activation on airway sensory nerves.

3.1.2 Aims

1. Investigate the effect of DSCG on the LAR response;
2. Confirm the effect of blocking TRPA1 in the LAR model;
3. Measure the impact of DSCG on TRPA1-driven stimulation of the sensory nerve activation *in vitro* and *in vivo*.

3.2 Methods

3.2.1 Ovalbumin (OVA)-Driven Rat Model of LAR

The OVA-driven rat model of LAR has been developed in the group previously²⁷. This is a model that mimics the allergic inflammatory response in asthmatics by using ovalbumin protein to sensitise and challenge the animals.

3.2.1.1 DSCG protocol

For the DSCG experiments, male Brown Norway rats were sensitised and challenged as described in 2.7.1. On the day of allergen challenge, rats were dosed with DSCG (10 mg/kg; 1 ml/kg; i.t.) or vehicle (0.5% v/v ethanol in saline, 1 ml/kg; i.t.) or budesonide (3 mg/kg; i.t.) 60 minutes before allergen challenge and 30 minutes following the end of allergen challenge^{236,237}. Penh was recorded from 60 minutes after the end of allergen challenge for a period of 5 hours, as detailed in general methods section 2.7.2.2.

3.2.1.2 Janssen 130 protocol

Male Brown Norway rats (200-225 g) were sensitised as detailed in general methods section 2.7.1. On the day of allergen challenge, rats were dosed with the TRPA1 antagonist Janssen 130 (100 or 300 mg/kg; i.p.) or vehicle (0.5% v/v ethanol in saline; i.p.) 60 minutes before allergen challenge and 30 minutes following the end of allergen challenge. Penh was recorded from 60 minutes after the end of allergen challenge for a period of 5 hours as detailed in general methods section 2.7.2.2.

3.2.1.3 Analysis

The Penh average was plotted against time following challenge for each group.

3.2.2 Isolated vagal nerve recordings

Vagal tissue was dissected from male Brown-Norway rats and Dunkin-Hartley guinea pigs and placed in the recording set up as previously described in Section 2.6.1. Key experiments were repeated with vagal nerve isolated from human tissue to generate translational data.

For experiments using the TRPA1 agonist acrolein as the test stimulus, an acrolein-driven vagal nerve depolarisation concentration response curve was first established. We then selected a submaximal dose of acrolein (300 μ M) for antagonist experiments.

3.2.2.1 TRPA1 antagonist concentration response curves

In order to confirm the effect of TRPA1 channel in vagal nerve depolarisation, a selective TRPA1 antagonist Janssen 130 was employed⁸⁶. Animal and human tissue were obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following this, TRPA1 agonist, acrolein (300 μ M) was perfused for two minutes twice to obtain reproducible responses. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline state. A third stimulation might be needed if reproducibility was not achieved. The nerve was then incubated with either vehicle (0.01% v/v DMSO) or TRPA1 antagonist Janssen 130 (0.01, 0.1, 1 or 10 μ M) for 10 minutes and acrolein was then reapplied in the presence of the antagonist for 2 minutes. The nerve was then washed as previously described and a final application of acrolein applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

3.2.2.2 DSCG concentration response curves

In order to determine the effect of DSCG on TRPA1-driven vagal nerve depolarisation, a concentration response experiment was conducted. The TRPA1 agonist acrolein (300 μ M) was employed in this set of experiments.

Animal and human tissue was obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. In rat tissue, 2 stimulations of acrolein (300 μ M) were perfused and repeatable response obtained. Between stimulations, Krebs solution was used to wash off the compound and return responses to baseline. The nerve was then incubated with either vehicle (0.1% v/v dH₂O) or DSCG (1, 10, 100 μ M) for 10 minutes and acrolein then reapplied in the presence of DSCG for 2 minutes. The nerve was then washed as previously described and a final application of acrolein (300 μ M) applied in order to test nerve viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial acrolein

responses. In guinea pig and human vagal nerves, only one concentration of DSCG (10 μ M) was investigated based on the results from the concentration response in the rat.

3.2.2.3 Investigation of the effects of DSCG on other tussive stimuli

To investigate the specificity of DSCG, agonists of TRPV1 (capsaicin; 1 μ M) and TRPV4 (GSK1016790a; 300 nM) were employed. 2 stimulations of TRPV1 agonist (capsaicin; 1 μ M) or TRPV4 agonist (GSK1016790a; 300 nM) were perfused and repeatable response obtained. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline. The nerve was then incubated with either vehicle (0.1% v/v dH₂O) or DSCG (10 μ M) for 10 minutes and the stimulant then reapplied in the presence of the drug for 2 minutes. The nerve was then perfused with Krebs as previously described and a final application of stimulating agent applied in order to test nerve viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial responses.

3.2.3 Intracellular calcium imaging in isolated airway vagal ganglia neurons

Airway-terminating neurons were stained using the retrograde neuronal tracer Dil (described in 2.3.1. 14 days after administering the dye, the ganglia neurons were extracted (described in 2.3.2) from male Brown Norway rat or Dunkin-Hartley guinea pig nodose and jugular ganglia. Cells were plated onto D-polylysine/Laminin-coated culture dishes and primary cultured for 24 hours with complete F12 medium to allow full adherence of the cells. For Calcium imaging the cells were loaded with 6 μ M Fura-2 acetoxymethyl ester (Fura-2 AM) for 40 minutes. To perform experiments, neuronal cells were rinsed and maintained in extracellular solution (ECS) to de-esterify for at least 30 min. Neurons were firstly observed by brightfield, and airway neurons identified by the presence of Dil fluorescence in individual neurons. Viability of the cells were assessed by applying 50 mM potassium solution (K50) at the start and end of experiments.

To study the role of DSCG in TRPA1-driven airway specific neuron activities, a concentration of TRPA1 agonist acrolein was selected as 10 μ M based on its pEC₅₀ value 5.3²²¹. Acrolein was applied twice for 30 seconds and washed until the calcium level returned to baseline. Vehicle (0.1% v/v dH₂O in ECS) or DSCG (10 μ M) was applied for 10 minutes and then acrolein reapplied in the presence of DSCG or vehicle. After a wash period, the acrolein was reapplied. Cell viability was assessed by a final response to K50. All recordings were performed at 37°C. Intracellular calcium change was recorded and analysed using HClmage and Image J (described in 2.3.3). Statistical difference was assessed on acrolein-induced responses using a paired t-test comparing with or without the presence of DSCG.

3.2.4 In vivo single fibre action potential firing recording

Guinea pigs were prepared as described in Section 2.6.2 and as previously described⁷³. Briefly, in order to specifically record airway sensory fibre firing upon stimulation and block any triggered feedback efferent firing, both vagal nerves were cut at the cervical end, and the left vagal nerve used for recording. The vagus nerve was dissected down to a single fibre and attached to a platinum electrode where single action potentials could be recorded using Spike 2 data acquisition software via a CED Micro 1401 interface. C-fibres were investigated in this experiment as acrolein was previously shown to only activate the chemosensitive C-fibres²³⁸. C-fibres were identified using a set of criteria⁷³ and as described in Section 2.6.2. This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, adaptation Indices, response to capsaicin and citric acid and finally conduction velocity.

3.2.4.1 DSCG response

After surgery the animals were allowed to stabilise for at least 30 min. Following identification of a lung afferent C-fibre and its sensitivity to capsaicin aerosol (capsaicin 100 μ M aerosol for 15s), the ensuing protocol was pursued: After a control baseline recording of at least 2 min, capsaicin (100 μ M) was administered by aerosol for 15 s and the changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. 2 control responses were obtained to aerosolised acrolein (15s, 10 mM) and after a suitable interval, either DSCG (30 mg/kg) or (1% v/v ethanol in saline) was administered i.t. to the animals and another response to acrolein obtained, 30 min after administration of DSCG.

3.2.4.2 Analysis

The data are expressed as mean $\pm$ S.E.M. and analysed using an unpaired t-test comparing responses between vehicle and DSCG treated animals.

3.2.5 Conscious guinea pig cough recording

Conscious, unrestrained guinea pigs were placed in individual transparent Perspex plethysmography chambers (Buxco, Wilmington NC, USA), as described in 2.7.1. Cough was detected as previously described⁸³. In all experiments, the researchers were blinded to the treatment groups.

3.2.5.1 TRPA1 antagonist concentration response

For the antagonist study, guinea pigs were injected intraperitoneally with Janssen 130 (30, 100 or 100 mg/kg) or vehicle (1% ethanol and 1% Tween 80, v/v, in saline) 1 hour before exposure. Acrolein (100

mM) was aerosolized for 5 minutes, and coughs were counted for 10 minutes by trained observers (2 in the room), and a mean of the 2 observer's counts was taken as the final count.

3.2.5.2 DSCG response

For the DSCG study, guinea pigs were anaesthetised with 4% (v/v) isoflurane and then intratracheally dosed with DSCG (100 mg/kg) or vehicle (saline) 30 minutes before acrolein exposure. Acrolein (100 mM) was aerosolised for 10 minutes, and coughs were counted for 10 minutes by trained observers (2 in the room), and a mean of the 2 observer's counts was taken as the final count.

3.2.5.3 Analysis

A Mann Whitney U test was employed to test inhibition of cough by the TRPA1 antagonist and DSCG, comparing responses between drug-treated groups to vehicle-treated group. The data is presented as mean $\pm$ S.E.M, with statistical significance set at $P < 0.05$.

3.3 Results

3.3.1 Effect of DSCG in ovalbumin (OVA)-driven late asthmatic response (LAR)

The first aim of my thesis was to determine if DSCG was able to attenuate the LAR in this OVA-driven rat model. Figure 3.1 shows average PenH recording over time. The group challenged with saline did not develop a response (Black trace). Whereas, following OVA challenge the vehicle treated group had a marked increase in PenH, indicating a clear Late Asthmatic Response (Purple trace). DSCG treatment significantly inhibited the LAR during the early phase of the LAR (Blue trace). The glucocorticoid budesonide (3 mg/kg), used as a positive control, successfully inhibited the LAR (Dark purple trace). This data has successfully shown that DSCG can inhibit the LAR in this BN rat model system.

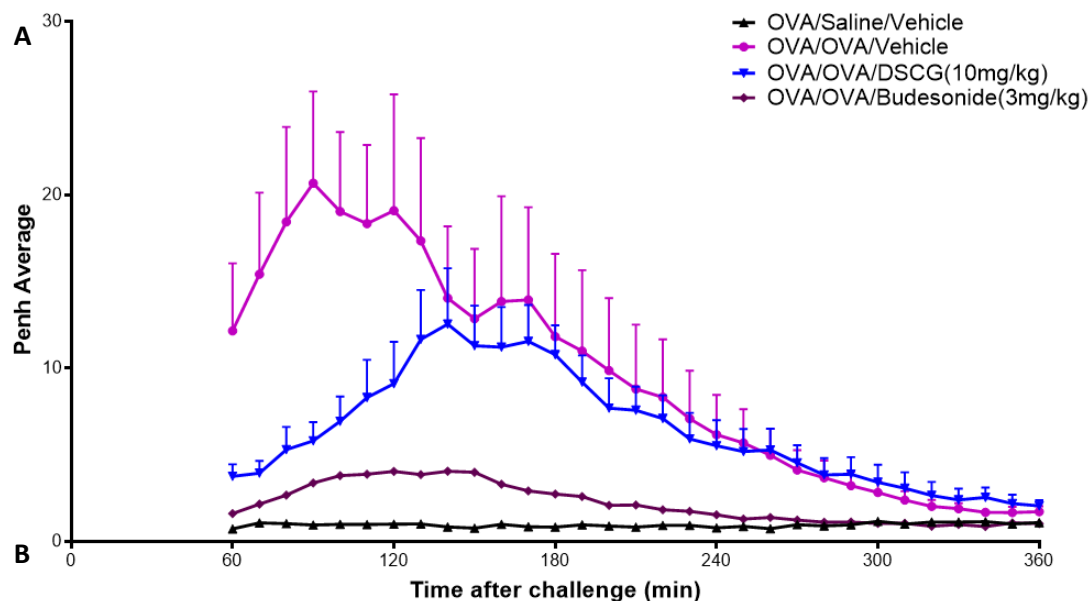


Figure 3.1 Effect of DSCG on OVA-driven LAR in rat

The effect of DSCG on OVA induced late asthmatic response at dose of 10 mg/kg. OVA/Saline/Vehicle stands for the group sensitised by OVA, challenged by Saline and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/Vehicle stands for the group sensitised by OVA, challenged by OVA and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/DSCG stands for the group sensitised by OVA, challenged by OVA and dosed by DSCG(10 mg/kg). OVA/OVA/Budesonide stands for the group sensitised by OVA, challenged by OVA and dosed by budesonide (3 mg/kg). Budesonide is included as a positive control. Penh was used as an indicator of lung function and arbitrary measure of constriction. Data is expressed as Penh average (per 10 min) over the recording time period. $n=8$.

3.3.2 Effects of TRPA1 in depolarisation of isolated vagal nerves

Having found that DSCG can inhibit the LAR in this model system, the next aim of my thesis was to confirm the role of TRPA1 in the same model. Previously it has been shown that the TRPA1 antagonist HC-030031 can inhibit the LAR²⁷, therefore I examined the effect of a selective, structurally different, TRPA1 antagonist, Janssen 130.

Before using Janssen 130 into the LAR model, I firstly wanted to confirm that the compound could inhibit TRPA1 ion channels on rat sensory nerves using a tissue based system (the isolated vagus). Previous studies have shown that the TRPA1 agonist, acrolein, causes a concentration-dependent increase in depolarisation of the isolated vagus nerve, and that the TRPA1 antagonist HC-030031 (10 μ M) significantly inhibits the response to acrolein⁸³. Janssen 130 was then profiled in this system to determine its effect against the previously determined sub-maximal concentration of acrolein (300 μ M).

Janssen 130 inhibited acrolein-induced depolarisation in a concentration-dependent manner in vagus nerves from BN rat, same species/strain used in the model of LAR (Figure 3.2A). Parallel studies were

also performed using guinea pig vagus tissue as this species is used for cough studies²⁰³ (rats do not possess the cough response²³⁹). Janssen 130 inhibited acrolein-induced response concentration-dependently in guinea pig vagal nerve (Figure 3.2B). Key experiments were performed using human tissue: Janssen 130 (10 μ M) was able to inhibit acrolein-induced depolarisation to 82.2% (Figure 3.2 C).

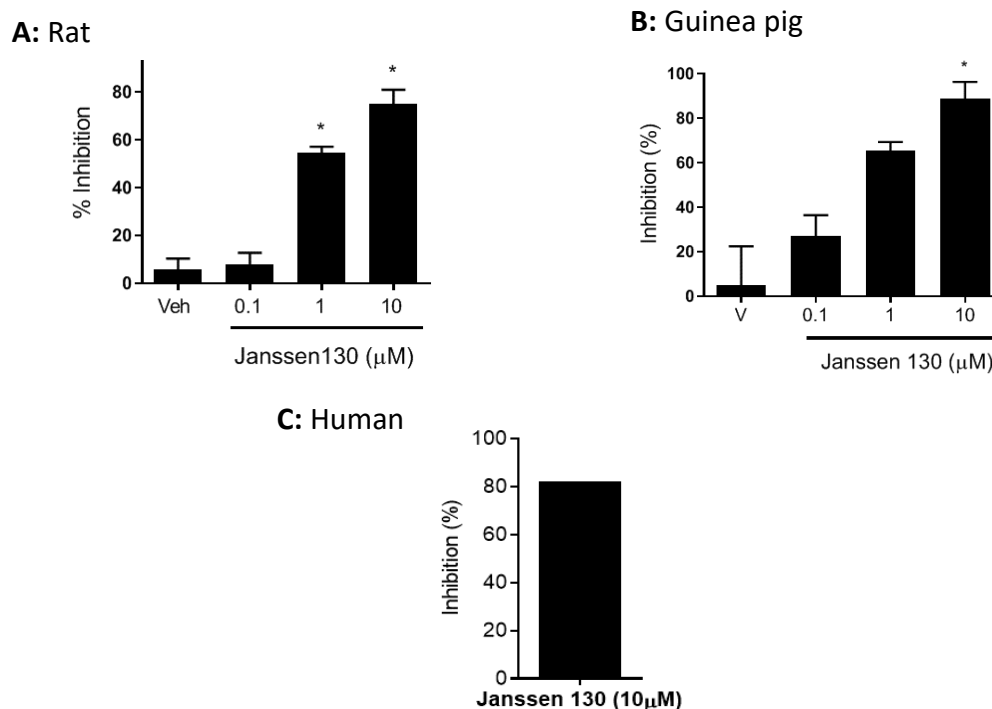


Figure 3.2 Effect of TRPA1 antagonist Janssen 130 on TRPA1 agonist acrolein induced depolarisation

Graphs showing the inhibitory effect of vehicle (0.1% v/v DMSO) or Janssen 130 (0.1-10 μ M) on (A) BN rat vagus nerve or (B) guinea pig vagus nerve stimulated with 300 μ M acrolein. (C) Graph showing the inhibitory effect of vehicle (0.1% v/v DMSO) or Janssen 130 (10 μ M) on human vagus nerve stimulated with 30 μ M acrolein. Data are expressed as percentage inhibition of acrolein internal control responses. The data is presented as mean $\pm$ S.E.M. of n=4 (A,B) and n=2 (C) observations. 2 donor patients: 2 males, 46-57 years old. * indicates $p < 0.05$ as determined by paired students t-test comparing responses in the presence of vehicle or antagonist to internal control responses within each group.

3.3.3 Effects of TRPA1 in ovalbumin (OVA)-driven late asthmatic response (LAR)

The next aim of my thesis was to confirm the role of TRPA1 in OVA-driven model of the LAR by using TRPA1 antagonist Janssen 130. There was no response in OVA-sensitised and saline-challenged rats. The TRPA1 antagonist Janssen 130 caused a dose-dependent reduction of the LAR response, with statistical significant inhibition at 300 mg/kg. This data adds further evidence to the concept that TRPA1 is involved in the LAR in this rodent model.

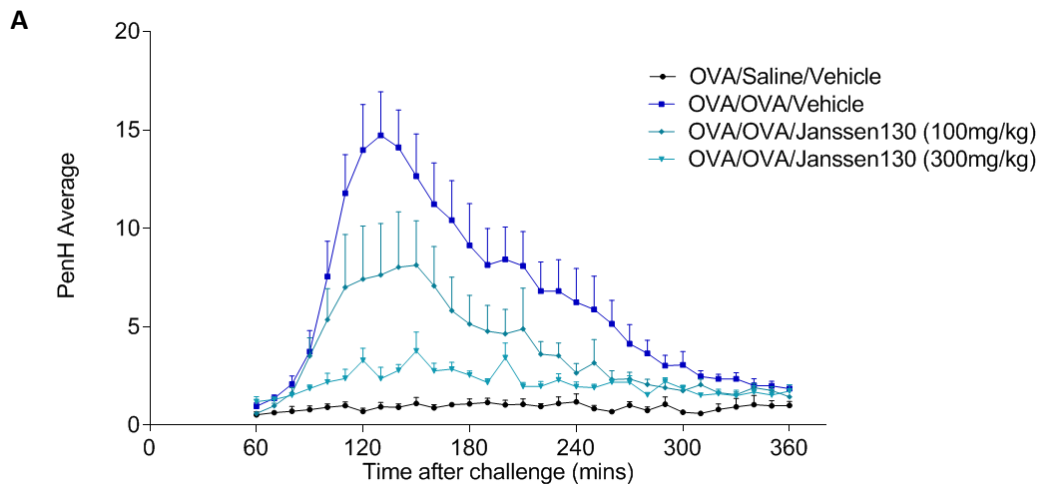


Figure 3.3 Effect of Janssen 130 on OVA-driven LAR in BN rat

The effect of TRPA1 antagonist Janssen 130 (100 mg/kg and 300 mg/kg) on OVA-induced LAR. OVA/Saline/Vehicle stands for the group sensitised by OVA, challenged by Saline and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/Vehicle stands for the group sensitised by OVA, challenged by OVA and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/Janssen stands for the group sensitised by OVA, challenged by OVA and dosed by Janssen 130 (100 mg/kg and 300 mg/kg). Penh was used as an indicator of lung function and arbitrary measure of constriction. Data is expressed as Penh average (per 10 min) over the recording time period. n=8.

3.3.4 Role of DSCG in TRPA1-driven depolarisation of isolated vagal nerves

I have shown that TRPA1 antagonist inhibits the LAR, suggesting a role for TRPA1 activation in the LAR. The same TRPA1 antagonist can inhibit TRPA1 activation of sensory nerves *in vitro*. DSCG can inhibit the LAR as well. Therefore, I want to investigate if DSCG has any effect on TRPA1 induced activation of airway sensory nerves. Isolated vagus nerves dissected from rats, guinea pigs and human tissue was investigated.

The impact of DSCG (1-100 μ M) on TRPA1 agonist acrolein-induced depolarisation was initially investigated in BN rat vagus tissue. DSCG inhibited acrolein-induced depolarisation in a concentration-dependent manner, demonstrating that DSCG is able to attenuate TRPA1 mediated activation of airway sensory nerves (Figure 3.4 A, B). A sub-maximal concentration of DSCG (10 μ M) was selected for further investigation and used in guinea pig and human tissue. To confirm the impact of DSCG on TRPA1-induced nerve activation, a second TRPA1 agonist AITC was used. DSCG also blocked AITC-induced vagus nerve depolarisation. (Figure 3.4, C).

To investigate whether this effect was specific to TRPA1 ion channels, agonists of TRPV1 (capsaicin; 1 μ M) and TRPV4 (GSK101; 300 nM) ion channels were employed. Unlike acrolein or AITC, DSCG did not inhibit depolarisation induced by TRPV1 or TRPV4 agonists. This data suggest that the inhibitory effect

of DSCG on sensory nerves is restricted to when the systems are triggered through the TRPA1 ion channel.

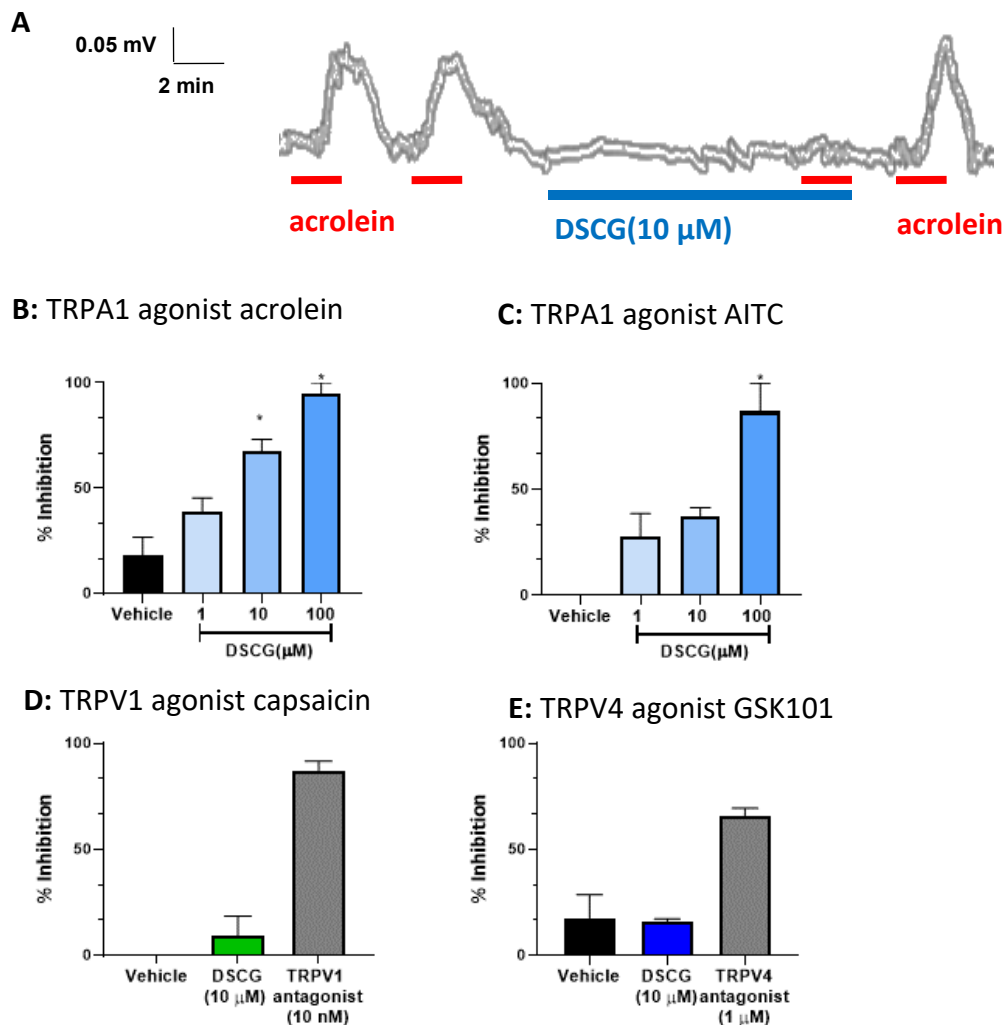


Figure 3.4 Effect of DSCG on TRPA1, TRPV1 and TRPV4 mediated depolarisation of BN rat vagus nerve

A) Example trace of DSCG (10 μ M) against TRPA1 agonist acrolein on rat vagus nerve. B) Graph showing effect of vehicle (0.1% v/v dH₂O) or DSCG (1, 10 or 100 μ M) on acrolein (300 μ M) induced depolarisation. C) Graphs showing effect of vehicle (0.1% v/v dH₂O) or DSCG on TRPA1 agonist AITC (100 μ M)-induced depolarisation. D) Effect of vehicle, DSCG or TRPV1 antagonist XEN-DO501 (10 nM) on TRPV1 agonist capsaicin (1 μ M)-induced depolarisation. E) Effect of vehicle, DSCG or TRPV4 antagonist GSK219 (1 μ M) on TRPV4 agonist GSK101 (300 nM)-induced depolarisation. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, n=4. *p<0.05 comparing depolarisation levels before and after treatment using a paired t-test.

Similar effect of DSCG on TRPA1 was found in other species. In the guinea pig, similarly to the rat, DSCG (10 μ M) was able to inhibit acrolein induced depolarisation, but not capsaicin induced depolarisation (Figure 3.5 A and B). In human vagal nerves, DSCG inhibited acrolein induced depolarisation but had no effect on capsaicin induced depolarisation (Figure 3.5 C). These two sets of

data indicate that, similarly to what was seen in the rat, DSCG is also able to specifically inhibit TRPA1 mediated activation of vagal nerves in the guinea pig and human tissue.

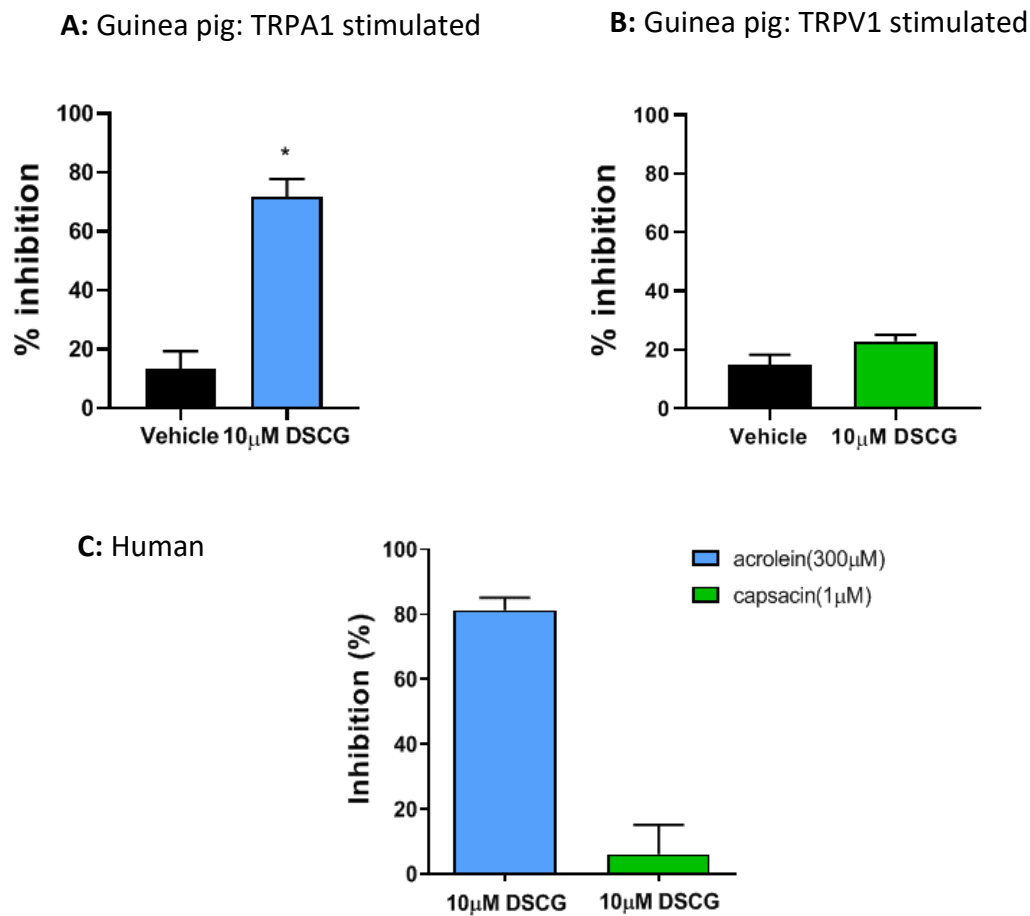


Figure 3.5 Effect of DSCG on TRPA1, TRPV1 mediated depolarisation of GP and human vagus nerve

A) Graph showing effect of vehicle (0.1% v/v dH₂O) or DSCG (10 μ M) on acrolein (300 μ M) induced depolarisation in GP vagus nerve. B) Graphs showing effect of vehicle (0.1% v/v dH₂O) or DSCG (10 μ M) on TRPV1 agonist capsaicin (1 μ M) induced depolarisation (C) Graphs showing effect of DSCG (10 μ M) on acrolein (300 μ M) and TRPV1 agonist capsaicin (1 μ M) induced depolarisation. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, n=4. 4 donor patients: 3 males, 36-57 years old and 1 female, 45 years old. * p <0.05 comparing depolarisation levels before and after treatment using a paired t-test.

3.3.5 Role of DSCG in TRPA1-driven calcium imaging on isolated vagal ganglia neurons

In 3.3.4, I have successfully indicated that DSCG is able to inhibit TRPA1-induced depolarisation in the isolated vagus nerve. Although it is a useful system, the vagus nerve not only innervates the respiratory system, but also innervates the heart and other tissues. To confirm that DSCG can inhibit lung-specific nerves, I performed experiments with airway ganglia neurons from both the rat and guinea pig.

Initially, it was determined that activation of the TRPA1 channel can lead to calcium influx in guinea pig airway-specific vagal ganglia neurons. The TRPA1 specific agonist, acrolein (10 μ M), was employed

to activate the TRPA1 channel, as this had been shown to activate the neurons previously⁸⁴(Figure 3.6). Example images and an example trace (Figure 3.6 A, B) indicated that acrolein was able to activate the neurons in a reproducible manner. This data is shown graphically in Figure 3.6 C. Of note, the vehicle for DSCG (0.1% v/v DMSO) had no effect on the intracellular calcium increases triggered by acrolein.

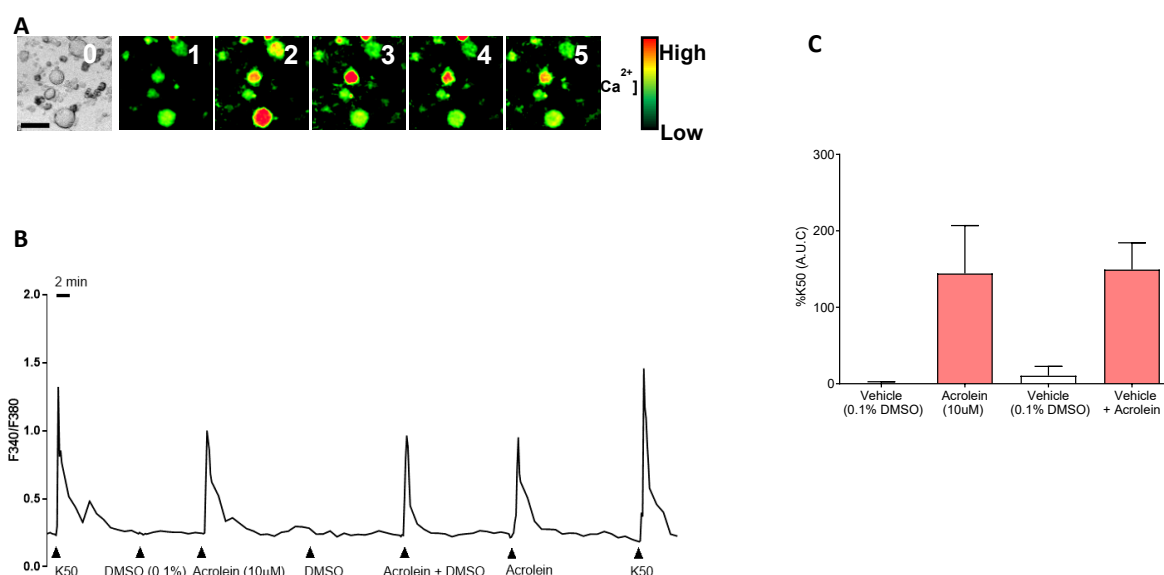


Figure 3.6 TRPA1 induced calcium-flux in guinea pig isolated airway specific neurons

A) Example of bright field and colour coded images of intracellular calcium levels in isolated airway neurons. Increase in red indicates increase of calcium with a colour indicator to the right. Picture number 1 to 5 on images indicates the corresponding point on the example trace below (0: Bright field; 1: Before treatment; 2: High potassium solution K50 (50mM potassium); 3: Acrolein (10 μ M); 4: Acrolein in presence of vehicle (0.1% v/v DMSO); 5: Acrolein). Scale bar is 50 μ m. B) The example trace of intracellular calcium levels induced by drug treatments was determined from analysis of images taken every 10s, and displayed as fluorescence ratio F_{340}/F_{380} with baseline corrected. C) Summary graph showing the effect of acrolein on intracellular calcium levels in neurons as determined by calculation of area under curve (AUC) from traces of each experiment. Data are presented as mean $\pm$ S.E.M. $n=5-19$ neurons from 2 guinea pigs.

After confirming that activation of TRPA1 channel can lead to calcium influx in airway-specific vagal ganglia neurons, the impact of DSCG on TRPA1-driven neuron activation was determined.

In figure 3.7, following acrolein (10 μ M) responses, DSCG (10 μ M) was incubated with airway jugular neurons from the guinea pig for 10 minutes. In the presence of DSCG, calcium influx by TRPA1 agonist acrolein was significantly reduced. This is indicated in example images (Figure 3.7A), example traces (Figure 3.7B) and graphically (Figure 3.7C). Following a wash step, the cells responded to acrolein indicating a viable preparation. This data was also repeated in rat airway neurons (Figure 3.8). This data suggest that the inhibitory effect of DSCG on TRPA1 channel activation occurs in airway-specific vagal ganglia sensory neurons.

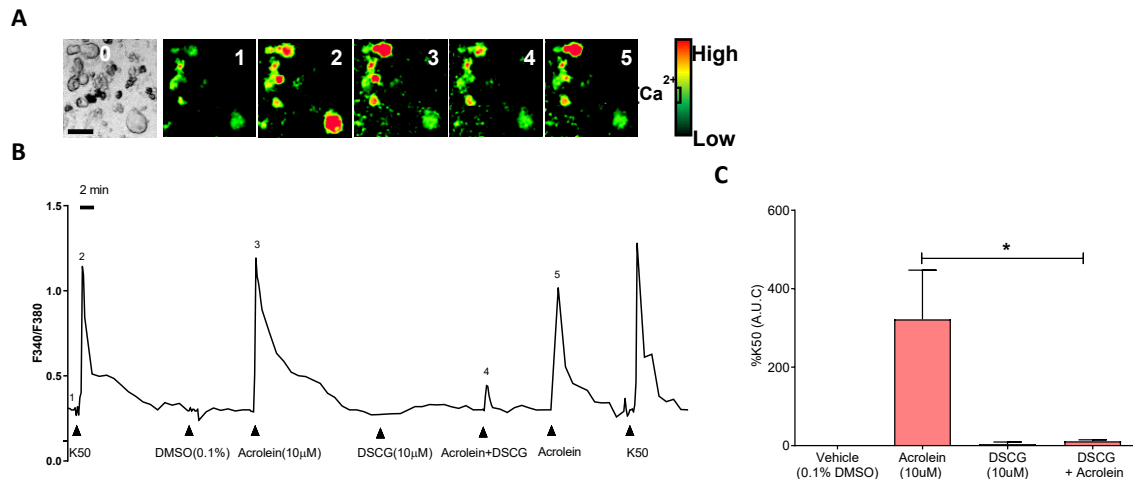


Figure 3.7 Effect of DSCG on TRPA1 induced calcium-flux in guinea pig isolated airway specific neuron

A) Example of bright field and colour coded images of intracellular calcium levels in isolated airway neurons. Increase in red indicates increase of calcium with a colour indicator to the right. Picture number 1 to 5 on images indicates the corresponding point on the example trace below (0: Bright field; 1: Before treatment; 2: High potassium solution K50 (50 mM potassium); 3: Acrolein (10 μM); 4: Acrolein in presence of DSCG (10 μM); 5: Acrolein). Scale bar is 50 μm. B) The example trace of intracellular calcium levels induced by drug treatments was determined from analysis of images taken every 10 seconds, and displayed as fluorescence ratio F340/F380 with baseline corrected.

C) Summary graph showing the effect of DSCG on acrolein-induced intracellular calcium levels in neurons as determined by calculation of area under curve (AUC) from traces of each experiment. Data are presented as mean ± S.E.M. n=5-19 neurons from 2 guinea pigs. *p<0.05 comparing calcium levels before and after treatment using a paired t-test.

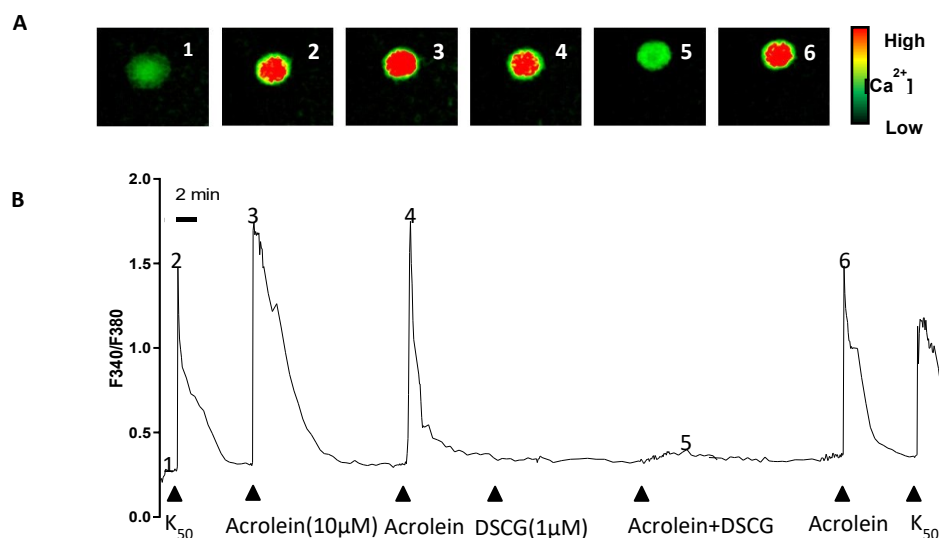


Figure 3.8 Effect of DSCG on TRPA1 induced calcium-flux in rat isolated airway specific neuron

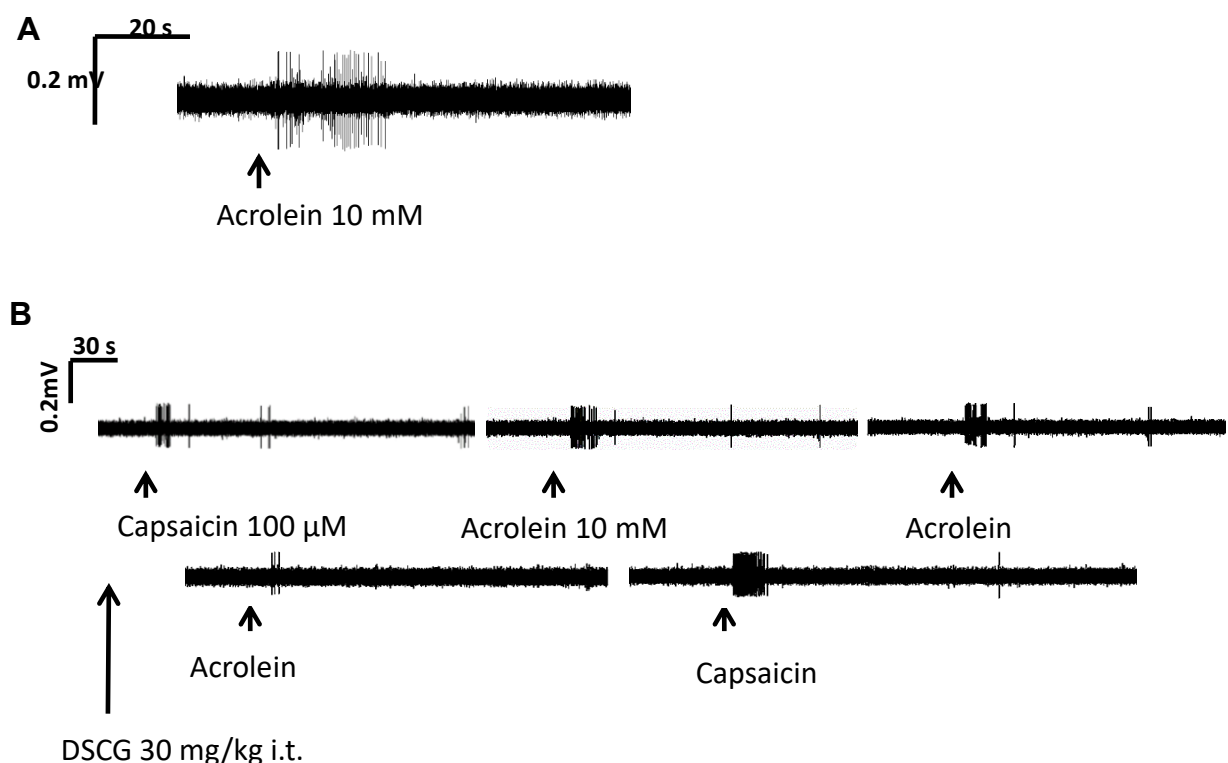
A) Example of bright field and colour coded images of intracellular calcium levels in isolated airway neurons. Increase in red indicates increase of calcium with a colour indicator to the right. Picture number 1 to 5 on images indicates the corresponding point on the example trace below (1: Before treatment; 2: High potassium solution

K50 (50 mM potassium); 3: Acrolein (10 μ M); 4: Acrolein in presence of DSCG (1 μ M); 5: Acrolein). B) The example trace of intracellular calcium levels induced by drug treatments was determined from analysis of images taken every 10s, and displayed as fluorescence ratio F340/F380 with baseline corrected.

3.3.6 Investigating the role of DSCG on TRPA1 driven airway single fibre firing

Having demonstrated the effect of DSCG on TRPA1-driven airway nerve activation *in vitro*, I next wanted to explore the effect in a whole animal preparation. To do this I selected an anaesthetised preparation in which one can record airway nerve firing recording in response to inhaled TRPA1 agonist.

As can be seen in Figure 3.9A, aerosolised acrolein¹⁴⁵ (10 mM) successfully triggered firing of afferent C fibres. To assess the impact of DSCG in this system it was dosed into the airways and subsequent acrolein-induced firing of the airway nerves was recorded. As can be seen in Figure 3.9, DSCG treatment significantly inhibited the TRPA1 signal. Capsaicin was applied at the start and end of the experiments to show selectivity of the effect of DSCG on TRPA1 channels and to demonstrate the viability of fibre after DSCG treatment. This data indicates that DSCG, when administered into the lungs, is able to inhibit TRPA1 triggered airway C-fibres *in vivo*.



C

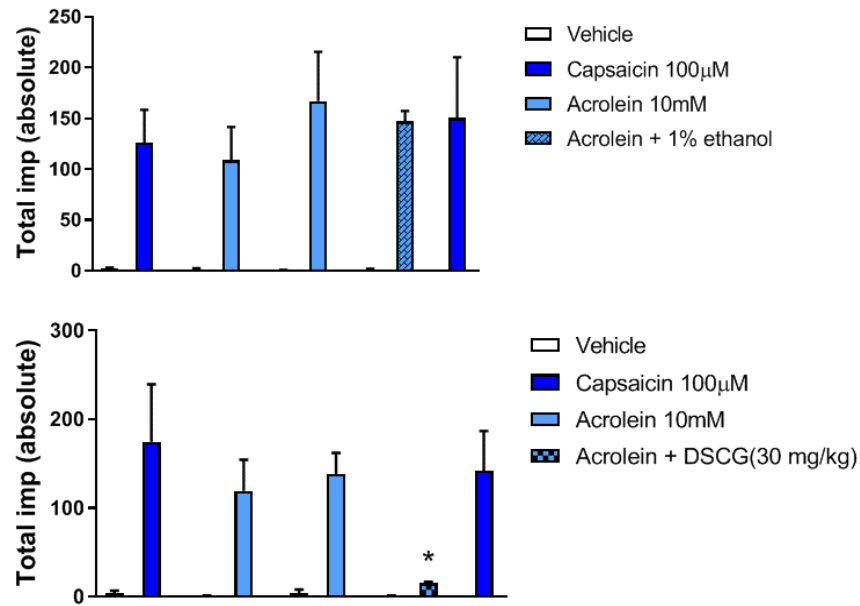


Figure 3.9 Effect of DSCG on acrolein (TRPA1) induced firing of C-fibre afferents in Guinea pig

A) Example trace of TRPA1 induced single fibre firing; B) Example trace of the effect of DSCG on TRPA1 induced single fibre firing. C) Two Panels show the total impulses before and after vehicle or DSCG application. Data is expressed as mean $\pm$ S.E.M. $n=3$. * $p<0.05$, paired t-test comparing before and after DSCG treatment.

3.3.7 Investigating the role of DSCG in conscious guinea pig cough

Finally, to further study the effect of DSCG on TRPA1 driven airway sensory nerve responses I utilised the cough reflex. Using a previous determined dose of inhaled acrolein, I was able to trigger coughing in the conscious guinea pig. Prior to evaluating the effect of DSCG, the role of TRPA1 was confirmed by using the same TRPA1 antagonist I used in the LAR model, Janssen130. As can be seen in Figure 3.10, Janssen 130 was able to cause a dose-dependent inhibition on cough evoked by acrolein. The level of inhibition, using the same doses, is similar to that seen in the LAR model.

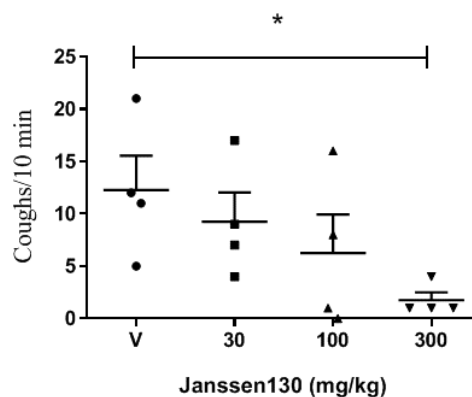


Figure 3.10 Effect of Janssen130 on TRPA1 agonist acrolein induced guinea pig cough model

*The effect of vehicle (0.5% methylcellulose, 0.2% tween 80 in saline, v/v) or Janssen130 (30, 100 or 300 mg/kg) on cough evoked by aerosol of acrolein (100 mM). Vehicle or Janssen130 were administered i.p. 60 min prior to 5 min aerosol challenge with acrolein. Coughs were counted for 10 min during aerosol challenge. Data are plotted as mean $\pm$ S.E.M of $n = 4$. * indicates $p < 0.05$ as determined by Kruskal-Wallis with Dunn's multiple comparisons test..*

Finally, DSCG was evaluated against cough evoked by acrolein. As can be seen in Figure 3.11, DSCG significantly reduced the number of coughs caused by acrolein compared to vehicle control. This result shows that DSCG can inhibit TRPA1 triggered airway sensory nerve reflexes.

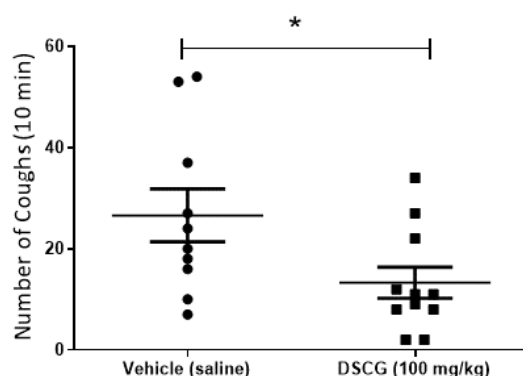


Figure 3.11 Effect of DSCG on TRPA1 agonist acrolein induced guinea pig cough model

*The effect of vehicle (saline) or DSCG (100 mg/kg) on cough evoked by aerosol of acrolein (100 mM). Vehicle or DSCG were administered i.t. 30min prior to 5 min aerosol challenge with acrolein. Coughs were counted for 10 min during aerosol challenge. Data are plotted as mean $\pm$ S.E.M of $n = 10$. * indicates $p < 0.05$ as determined by Mann-Whitney U-test.*

3.4 Discussion

DSCG is a historical asthma drug which shows effectiveness against a number of asthma symptoms both clinically and pre-clinically and which has a favourable safety profile¹⁶¹. Originally classed as a mast cell stabiliser, it has since been thought that the beneficial effects of DSCG are unlikely to be via this mechanism, as other mast cell stabiliser compounds have not shown the same favourable effects. The aim of this chapter was to model the effects of DSCG in a pre-clinical system and then to begin to investigate the mechanism by which it acts.

Initially, the effect of DSCG in OVA driven LAR was investigated. This involved using OVA protein to sensitise and challenge rats to trigger a LAR. In human asthmatic patients, the LAR typically occurs 3-8 hours following allergen exposure²⁷. The rat model I used exhibited a response peaking at 2-3 hours, lasting for up to 6 hours after challenge, mimicking the human clinical presentation of LAR symptoms. The results showed that DSCG inhibited the LAR, indicating that I could mimic the effect of DSCG in asthmatics and this system was relevant to investigate its mechanism of action.

Previous studies in the group have shown that in this rat model, allergen challenge triggers airway sensory nerves via the activation of TRPA1 channels on sensory nerves, triggering a parasympathetic reflex event which results in a cholinergic driven bronchoconstriction response²⁷. In this chapter this previous data was expanded on by utilising a second, structurally different, selective TRPA1 antagonist, Janssen 130. Similarly to DSCG, Janssen130 was found to significantly inhibit the LAR response in the BN rat, providing further confirmation that activation of the TRPA1 ion channel is likely to be a key driver of the LAR.

Previous data suggested that DSCG was able to inhibit TRPA1 induced activation of nerves from the GI tract²³⁵. Having shown that TRPA1 is required for the LAR response, and that DSCG can inhibit the LAR, this led me to postulate that DSCG may attenuate asthma symptoms by modulating TRPA1 on airway sensory nerves. To test this hypothesis, I wanted to determine whether DSCG could block TRPA1 activation of airway sensory nerves using a range of systems. Initially I employed the isolated vagus nerve preparation because it is a more amenable technique to begin my investigations and that human tissue can be used to translate my findings. Isolated vagal nerves from rats and guinea pigs were assessed; rats as these were the species used in the LAR model, and guinea pigs as they are the only small rodent that possesses a cough response. In order to determine the effect of DSCG on TRPA1 mediated activation, we first needed to confirm that acrolein induced depolarisation was caused by selectively opening the TRPA1 channel. In a previous study⁸³, the TRPA1 antagonist HC-030031 (10 μ M) blocked acrolein-driven vagal nerve depolarisation and to confirm this conclusion, a second TRPA1 antagonist, Janssen130, was employed. Janssen 130 inhibited depolarisation induced by the TRPA1 agonist acrolein (300 μ M) in rat, guinea pig and human isolated vagal nerves, confirming the role of TRPA1 in vagal nerve activation.

Having established TRPA1 driven responses, I used the system to investigate the effect of DSCG. Initially carried out in rat vagus nerves, DSCG caused a concentration dependent inhibition of both acrolein and AITC induced depolarisation. DSCG also inhibited acrolein induced depolarisation in guinea pig and in human isolated vagus nerves, indicating that this effect is not species specific. Interestingly, DSCG had no effect on either TRPV1 (capsaicin) or TRPV4 (GSK1016790a) induced depolarisation, suggesting the inhibitory effect observed was specific to TRPA1. This both suggests a novel mechanism of action for DSCG beyond its known function as a mast cell stabiliser, and also further implicates TRPA1 as a key driver in asthma symptoms such as the LAR.

Whilst the inhibitory effect of DSCG on TRPA1-mediated nerve activation was demonstrated in isolated whole vagus nerve segments, it is known that the vagus nerve innervates not only the airways, but also other visceral organs²⁴⁰. Therefore, in order to clarify that DSCG exerts its inhibitory effect on

TRPA1 on airway sensory nerves, vagal ganglia neurons specifically innervating the airway were isolated and intracellular calcium levels assessed as a marker of neuron activation. Acrolein was able to induce increases in intracellular Ca^{2+} in airway specific neurons, and this activation by acrolein was inhibited by DSCG in both guinea pig and rat airway neurons. These results demonstrated that DSCG can inhibit TRPA1 initiated airway nerves.

By using *in vitro* techniques to evaluate DSCG, I confirmed its inhibitory effects on TRPA1-driven airway nerve activities. However these techniques were only investigating *in vitro* functions, and neither assesses the effect of DSCG on a nerve terminal (in the airway) nor actual nerve firing. The expression of receptors and signal transduction pathways on the nerve trunk and/or ganglia cell bodies may not be the same as on the nerve endings. Therefore to further investigate this I employed two *in vivo* systems: afferent airway sensory nerve recording from anaesthetised animals and induced cough in the conscious guinea pig. Acrolein aerosolised into the lungs of anaesthetised animals triggers firing of C-fibres. This firing was significantly inhibited following i.t. administration of DSCG. This data confirms that DSCG, when applied at nerve terminals, is capable of inhibiting action potential firing induced by TRPA1 in GP C-fibres *in vivo*. Similarly, aerosolised acrolein triggers coughing in conscious guinea pigs. The data showed that pre-treatment with the selective TRPA1 antagonist, Janssen 130, or DSCG significantly inhibited the cough response. The anti-tussive impact of DSCG has been reported by others^{189,241,179}.

The results in this chapter have suggested that DSCG attenuates the LAR via inhibition of TRPA1 ion channels on airway sensory nerves. I can therefore accept my chapter hypothesis 'DSCG may attenuate asthma symptoms by modulating TRPA1 on airway sensory nerves'. It would appear that DSCG is not a direct TRPA1 channel blocker, therefore the next aim was to determine how DSCG could inhibit this ion channel. A previous study has reported that DSCG is a potent agonist of the g-protein coupled receptor 35 (GPR35)^{191,192}. Furthermore, GPR35 agonists have been suggested to regulate calcium signaling in neurons¹⁹⁴. Therefore, my next chapter investigates the role of GPR35 in DSCG-induced inhibition of TRPA1 sensory nerve activation.

Chapter 4 Investigation into the mechanism by which DSCG inhibits airway sensory nerves

4.1 Rationale

In Chapter 3 I showed that DSCG can attenuate the LAR in a rodent model of allergic asthma and it could reduce TRPA1-induced activation on airway sensory nerves. The mechanism of action however remains undetermined; it appears that DSCG is not a direct blocker of the TRPA1 ion channel. The aim of this chapter was to determine how DSCG is exerting its inhibitory activity on TRPA1.

A previous study has shown that DSCG is a potent agonist of the G-Protein Coupled Receptor 35 (GPR35)^{191,192}. GPR35 is a G protein-coupled receptor that is encoded by the GPR35 gene in humans. G protein-coupled receptors are one of the biggest cell surface protein families composed of over 1000 members and making 3% of the human genome. It is known that GPCRs are involved in the mechanism of action of a wide range of therapeutics¹⁹⁴. Although ligands have been identified for many GPCRs there are still more than 100 GPCRs with no endogenous ligand known as yet and which are called orphan receptors. Orphan receptors may provide opportunities for drug discovery as they could be involved in signaling pathways and disease control/progression. Therefore, there is a major drive to identify agonists, antagonists and signal transduction pathways for these orphan GPCRs as they could lead to the identification of new drug targets.

The GPR35 receptor is one of these orphan receptors which are not well characterised. It has been found to be expressed in some regions of the central and peripheral nervous system, such as caudate nucleus in human, medulla oblongata, hippocampus, spinal cord and dorsal root ganglia in mouse^{225,242}, and dorsal root ganglia, spinal cord, hippocampus and cerebrum in rat^{243–246}. GPR35 agonists have been reported to regulate calcium signaling in neurons¹⁹⁴. Thus, I hypothesised that DSCG modulates the TRPA1-driven sensory nerve activity via activation of GPR35.

To test this hypothesis, I initially wanted to use the isolated vagus nerve preparation and a range of pharmacological tools. By using selective GPR35 agonists and antagonists, I hope to determine if GPR35 activation could replicate the effect of DSCG and whether GPR35 antagonists could block the impact of DSCG. I then used calcium imaging of airway-specific neurons *in vitro* to confirm that the observed functional effects were present in airway innervating neurons. Following this I will investigate the hypothesis using the *in vivo* systems used in chapter 3.

4.1.1 Chapter hypothesis

DSCG activates the GPR35 receptor to modulate TRPA1 activity on airway sensory nerves.

4.1.2 Aims

1. Investigate the role of GPR35 in the TRPA1-driven sensory nerve activation.

2. Investigate the effect of GPR35 in DSCG inhibition of the sensory nerve activation *in vitro* and *in vivo*.
3. Determine the effect of GPR35 in DSCG inhibition of the LAR.

4.2 Methods

4.2.1 Isolated vagal nerve recordings to assess GPR35 ligands

As described in the methods chapter in Section 2.6.1, vagal tissue was dissected from male Brown-Norway rats and Dunkin-Hartley guinea pigs and placed in the recording set up. Key experiments were repeated with vagal nerve isolated from human tissue to generate translational data.

As mentioned in method 3.2.2, for experiments using the TRPA1 agonist acrolein as the test stimulus, a submaximal dose of acrolein (300 μ M) was selected.

4.2.1.1 GPR35 agonist TC-G 1001 response curve

In order to investigate the effect of GPR35 on TRPA1 driven vagal nerve depolarisation, a selective GPR35 agonist TC-G 1001 was used²²⁴. Animal tissues were harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following this, the TRPA1 agonist acrolein (300 μ M) was perfused and washed twice to obtain 2 similar responses and ensure reproducibility. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with either vehicle (0.01% v/v DMSO) or GPR35 agonist TC-G 1001 (0.001, 0.01, 0.1, 1, 10 or 100 μ M) for 10 minutes and acrolein was then reapplied in the presence of the vehicle or TC-G 1001. In guinea pig and human vagal nerves, only one concentration of TC-G 1001 (10 μ M) was investigated based on the results from the concentration response in the rat. The nerve was then washed as previously described and a final application of stimulating agent applied in order to test nerve recovery and viability. A change in the level of depolarisation following test compound application was expressed as percentage of the average of the two initial basal responses.

To confirm the effect of GPR35, other two GPR35 agonists, kynurenic acid (10 μ M) and zaprinast (10 μ M), were applied following the same protocol in rat, guinea pig and human tissue^{225,226}.

4.2.1.2 Confirming the role of GPR35 utilising the selective antagonist ML145

To confirm the effect above is through GPR35 activation, a selective GPR35 antagonist ML145 was used²⁴⁷. Animal and human tissue were obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. As shown in Figure 4.1, TRPA1 agonist, acrolein (300 μ M) was perfused and washed twice

to obtain 2 similar responses and ensure repeatability. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with vehicle (0.1% v/v DMSO) or the GPR35 inhibitor ML145 (1 μ M) for 10 minutes. Following this the nerve was then incubated with either vehicle (0.1% v/v DMSO) or GPR35 agonist TC-G 1001 (10 μ M) for 10 minutes and acrolein was then reapplied in the presence of the antagonist. The nerve was then washed as previously described and a final application of stimulating agent applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses. A second GPR35 antagonist CID 2745687²⁴⁷ (1 μ M) followed the same protocol.

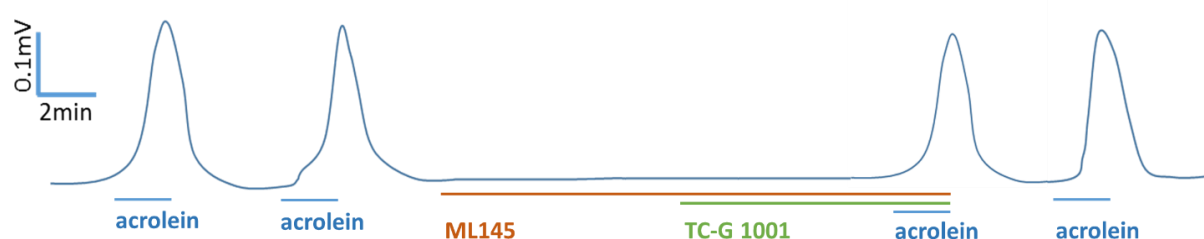


Figure 4.1 Example illustration of vagus nerve depolarisation recording protocol to measure the effect of GPR35 antagonist ML145 on the inhibitory activity of GPR35 agonist TC-G 1001

4.2.2 Single cell PCR for GPR35 gene expression in vagal ganglia

Quantitative real time PCR assays for GPR35 receptor were purchased from Applied Biosystems and validated using cDNA from tissue of guinea pig that expressed the target channel at high levels. Individual airway-specific jugular and nodose ganglia neurons were harvested from male Dunkin Hartley guinea pigs as described in Section 2.3.3. Expression levels of GPR35 were determined using the validated assays, as described in Section 2.3.3.

4.2.3 Intracellular calcium imaging in isolated airway specific vagal ganglia neurons

Airway-terminating neurons were stained using intranasal administration of the retrograde neuronal tracer Dil (described in 2.3.1). 14 days after administering the dye, the ganglia neurons were extracted (described in 2.3.2) from male Brown Norway rat or nodose and jugular ganglia from male Dunkin-Hartley guinea pigs. Cells were plated onto D-polylysine/Laminin-coated culture dishes and primary cultured for 24 hours with complete F12 medium to allow full adherence of the cells. For Calcium imaging the cells were loaded with 6 μ M Fura-2 acetoxymethyl ester (Fura-2 AM) for 40 minutes, rinsed with extracellular solution (ECS) and allowed to de-esterify for a further 30 minutes. Neurons were selected in Brightfield mode and airway neurons identified by the presence of Dil fluorescence. Viability of the cells were assessed by applying 50 mM potassium solution (K50) at the start and end

of experiments. To study the effect of GPR35 in TRPA1-driven airway specific neuron activities, a concentration of the TRPA1 agonist acrolein was selected as 10 μ M based on previous study in our group²²⁴. Acrolein was applied twice for 30 seconds and washed until the calcium level returned to baseline. Vehicle (0.1% v/v DMSO in ECS) or TC-G 1001 (10 μ M) was applied for 10 minutes and then acrolein reapplied in the presence of TC-G 1001 or vehicle. After a wash period, the acrolein was reapplied. Cell viability was assessed by a final response to K50. All recordings were performed at 37°C. Intracellular calcium change was recorded and analysed using HClImage and Image J (described in 2.3.3). Statistical difference was assessed on acrolein-induced responses using a paired t-test comparing with or without the presence of TC-G 1001.

4.2.4 Isolated vagal nerve recordings to assess the role of GPR35 in the inhibitory effect of DSCG

In order to investigate the effect of GPR35 in DSCG mediated inhibition of TRPA1 driven depolarisation in the isolated vagus nerve, a selective GPR35 antagonist ML145 was applied. Animal and human tissue were obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. As shown in Figure 4.2, TRPA1 agonist, acrolein (300 μ M) was perfused and washed twice to obtain 2 similar responses and ensure repeatability. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with vehicle (0.1% v/v DMSO) or the GPR35 inhibitor ML145 (1 μ M) for 10 minutes. Following this the nerve was then incubated with DSCG (10 μ M) for 10 minutes in presence of the vehicle or ML145. Acrolein was then reapplied in the presence of the drugs. The nerve was then washed off and stimulating agent acrolein was applied again in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses. A second GPR35 antagonist CID 2745687 (1 μ M) follows the same protocol.

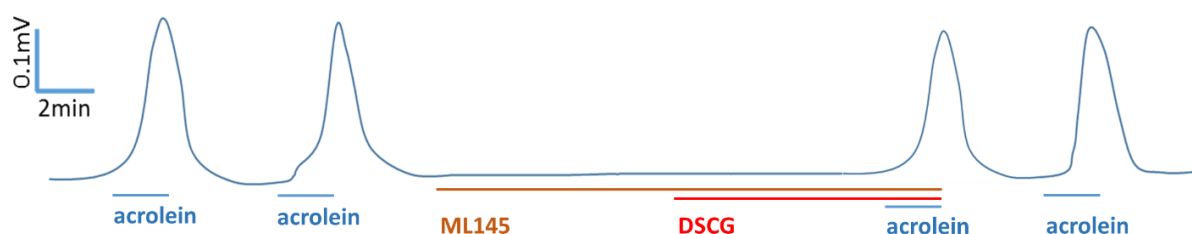


Figure 4.2 Example illustration of vagus nerve depolarisation recording protocol to measure the effect of GPR35 antagonist ML145 on the inhibitory activity of DSCG

4.2.5 *In vivo* single fibre action potential firing recording

Guinea pigs were prepared as described in Section 2.6.2 and as previously described⁷³. Briefly, in order to specifically record airway sensory fibre firing upon stimulation and block any triggered feedback efferent firing, both vagal nerves were cut at the cervical end, and the left vagal nerve used for recording. The vagus nerve was dissected down to a single fibre and attached to a platinum electrode where single action potentials could be recorded using Spike 2 data acquisition software via a CED Micro 1401 interface. C-fibres were investigated in this experiment as acrolein was previously shown to only activate the chemosensitive C-fibres²³⁸. C-fibres were identified using a set of criteria⁷³ and as described in Section 2.6.2. This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, adaptation Indices, response to capsaicin and citric acid and finally conduction velocity.

After surgery the animals were allowed to stabilise for at least 30 min. Following identification of a lung afferent C-fibre and its sensitivity to capsaicin aerosol (capsaicin 100 μ M aerosol for 15s), the ensuing protocol was pursued: After a control baseline recording of at least 2 min, capsaicin (100 μ M) was administered by aerosol for 15 s and the changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. 2 control responses were obtained to aerosolised acrolein (15s 10 mM) and after a suitable interval, vehicle (0.5% MC + 0.2% tween80 in saline, v/v; 10 ml/kg, i.p.) or GPR35 receptor antagonist, ML145 (30 mg/kg, i.p.) were administered. 30minutes following that, either DSCG (30 mg/kg, i.t.) or (1% v/v ethanol in saline; i.t.) was administered to the animals. 30 min later, another response to acrolein was obtained. The data are expressed as mean $\pm$ S.E.M. and analysed using an unpaired t-test comparing responses between vehicle and drug treated animals.

4.2.6 Ovalbumin (OVA) Driven Rat Model of LAR

The (OVA) driven rat model of LAR has been developed in the group previously²⁷. This is a model that mimics the allergic inflammatory response in asthmatics by using ovalbumin protein to sensitise and challenge the animals. For the GPR35 antagonist ML145 experiments, male Brown Norway rats were sensitised and challenged as described in 2.7.1. On the day of allergen challenge, rats were dosed with ML145 (30 mg/kg; 1 ml/kg; i.p.) or vehicle (0.5% methylcellulose + 0.2% tween80 in saline, v/v; 10 ml/kg, i.p.) 90 minutes before and 5 minutes after allergen challenge. The rats were dosed with DSCG (10 mg/kg; 1 ml/kg; i.t.) or vehicle (0.5% v/v ethanol in saline, 1 ml/kg; i.t.) 60 minutes before and 30minutes after allergen challenge. Dosing regimens were based on those used in previous studies^{236,237}. Penh was recorded from 60minutes after the end of allergen challenge for a period of 5 hours as detailed in general methods section 2.7.2. The Penh average was plotted against time

following challenge. Penh average at peak response for the various experimental groups was calculated. Mann Whitney U test were carried out to compare data between with and without drug treatment, with statistical significance set at $P < 0.05$.

4.3 Results

4.3.1 Effect of GPR35 receptor in TRPA1-mediated depolarisation of isolated vagus nerves

In order to test the hypothesis that DSCG modulated TRPA1-driven sensory nerve effects via the activation of GPR35, a range of GPR35 agonists were applied to the isolated vagus nerve to see if they could mimic the effect of DSCG on TRPA1-driven depolarisation.

A concentration response of the GPR35 agonist TC-G 1001²²⁴ (1 nM to 100 μ M) against TRPA1 agonist acrolein (300 μ M) induced depolarisation was initially carried out in the rat vagal nerve tissue. As shown on the trace and the graph (Figure 4.3 A and B) GPR35 agonist TC-G 1001 inhibited acrolein driven vagal nerve depolarisation in a concentration-dependent manner, with a maximum inhibition of $88.3 \pm 0.9\%$ at 100 μ M, suggesting that a GPR35 agonist is able to attenuate TRPA1-mediated activation of airway sensory nerves similarly to DSCG. A sub-maximal concentration of 10 μ M TC-G 1001 was selected for further investigation, and for confirmation in guinea pig and human tissue.

I used two other GPR35 agonists to confirm the data obtained with TC-G 1001 (Figure 4.3, C). Both kynurenic acid²²⁵ (10 μ M) and zaprinast²²⁶ (10 μ M) inhibited acrolein driven vagal nerve depolarisation by $64.8 \pm 17.7\%$ and $74.1 \pm 2.4\%$ respectively, confirming that stimulation of GPR35 lead to attenuation of TRPA1 mediated activation of airway sensory nerves in rat. They were applied for confirmation in guinea pig and human tissue.

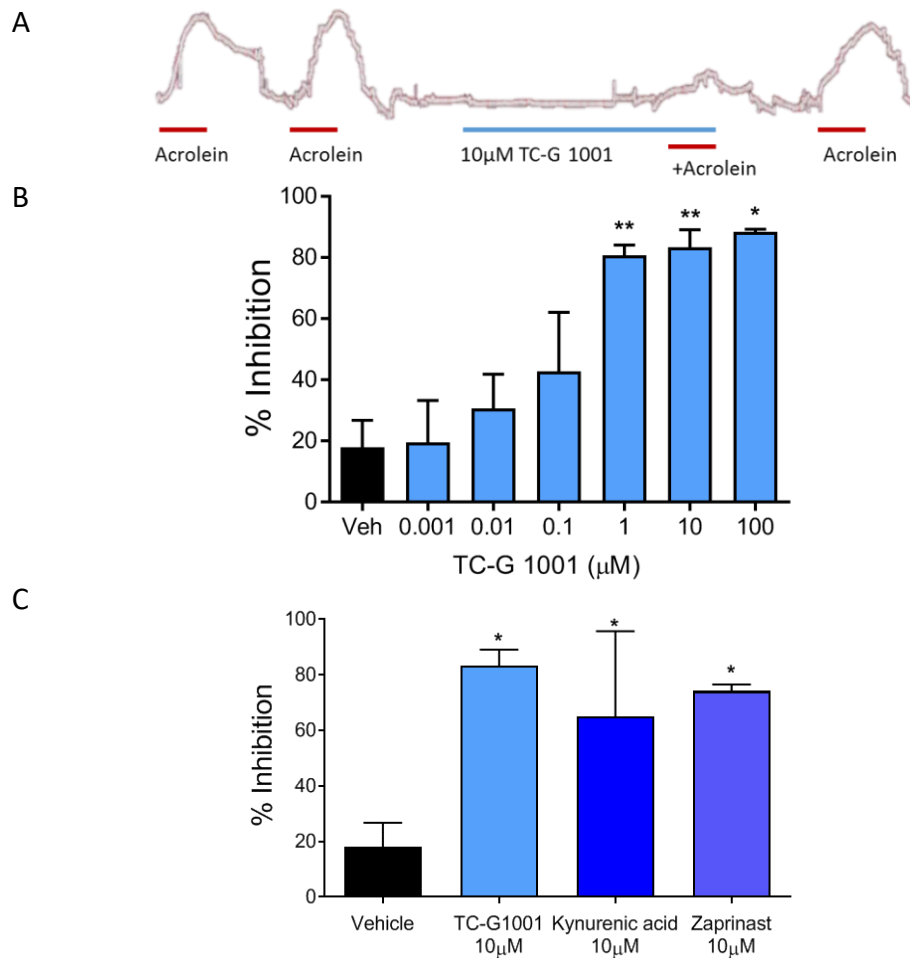
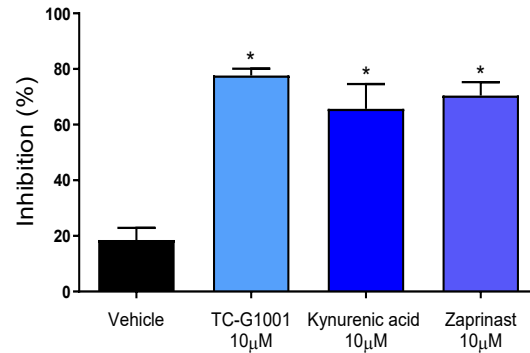


Figure 4.3 Effect of GPR35 agonist on TRPA1 agonist acrolein induced depolarisation in rat

A) Example trace of TC-G1001 (10 µM) against TRPA1 agonist acrolein on rat vagus nerve. B) Graph showing effect of vehicle (0.1% v/v DMSO) or TC-G1001 (1 nM to 100 µM) on acrolein (300 µM) induced depolarisation in rat vagus nerve. C) Graph showing effect of vehicle (0.1% v/v DMSO), TC-G1001 (10 µM), kynurenic acid (10 µM) or zaprinast (10 µM) on acrolein (300 µM) induced depolarisation in rat vagus nerve. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, $n=4$. * $p<0.05$; ** $p<0.01$ comparing depolarisation levels before and after treatment using a paired t-test

In figure 4.4, key experiments have been repeated in the guinea pig and in human tissue. In the guinea pig, similarly to the rat, GPR35 agonists TC-G 1001 (10 µM), kynurenic acid (10 µM) and zaprinast (10 µM) were able to significantly inhibit TRPA1 agonist acrolein-induced depolarisation by $77.7 \pm 2.4\%$, $65.6 \pm 8.9\%$ and $70.4 \pm 4.8\%$ respectively (Figure 4.4 A). In human vagal nerves, all three GPR35 agonists TC-G 1001 (10 µM), kynurenic acid (10 µM) and zaprinast (10 µM) significantly inhibited acrolein induced depolarisation by $88.3 \pm 5.8\%$, $78.7 \pm 14.9\%$ and $73.6 \pm 13.3\%$ respectively (Figure 4.4 B). These results indicate that, similar to observations in rat vagus, the GPR35 agonists (TC-G 1001, kynurenic acid and zaprinast) are able to significantly inhibit TRPA1 mediated activation of vagal nerves in guinea pigs and humans.

A: Guinea pig



B: Human

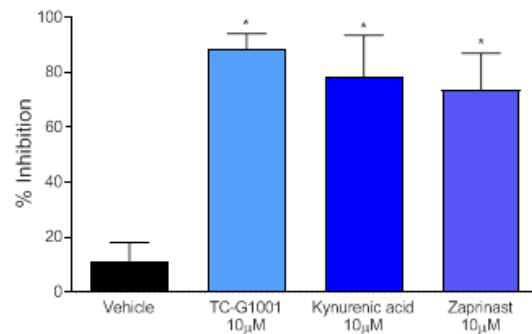
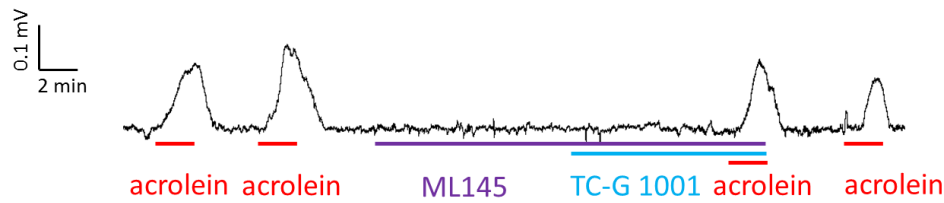


Figure 4.4 Effect of GPR35 agonists on TRPA1 agonist acrolein induced depolarisation in guinea pig and human

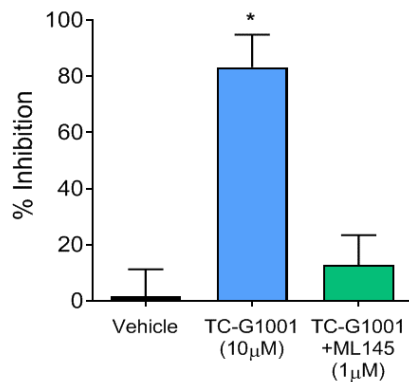
A) Graph showing effect of vehicle (0.1% v/v DMSO), TC-G 1001 (10 µM), kynurenic acid (10 µM) or zaprinast (10 µM) on acrolein (300 µM) induced depolarisation in guinea pig vagus nerve. B) Graph showing effect of vehicle (0.1% v/v DMSO), TC-G 1001 (10 µM), kynurenic acid (10 µM) or zaprinast (10 µM) on acrolein (300 µM) induced depolarisation in human vagus nerve. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, n=4. 4 donor patients: 3 males, 36-57 years old and 1 female, 45 years old. * $p < 0.05$ comparing depolarisation levels before and after treatment using a paired t-test

After evaluating the effect of GPR35 agonists on acrolein induced depolarisation, the GPR35 antagonist ML145 was then used to determine if the inhibition induced by GPR35 agonists at this concentration was GPR35-specific. As can be seen in Figure 4.5, the GPR35 antagonist ML145 (1 µM) was able to fully reverse the inhibitory effect of GPR35 agonist TC-G 1001 (10 µM) in rats and humans vagus nerve albeit only partially in guinea pig. This suggests that the TC-G 1001 inhibited acrolein-induced depolarisation specifically via GPR35 in rat and human. A second GPR35 antagonist CID 2745687 (1µM) was used for confirmation in guinea pig (Figure 4.5 B), which was able to fully reverse the inhibitory effect of GPR35 agonist TC-G 1001.

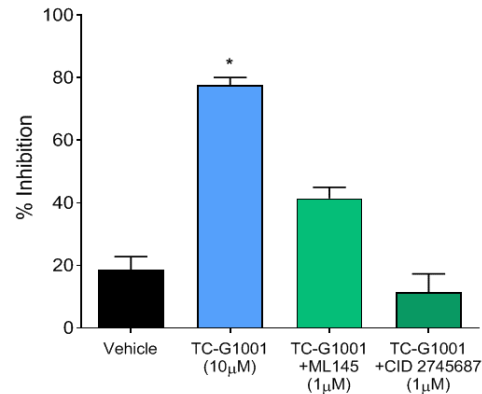
A: Guinea pig



B: Rat



C: Guinea pig



D: Human

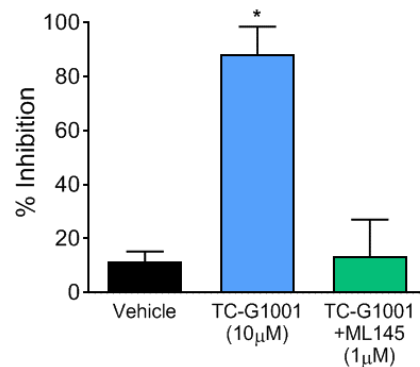


Figure 4.5 Effect of GPR35 antagonists on GPR35 agonist mediated inhibition of TRPA1 driven depolarisation

A) Example trace of GPR35 antagonist ML145 (1 µM) against GPR35 agonist TC-G 1001-mediated inhibition of TRPA1 agonist acrolein on guinea pig vagus nerve. B) Graph showing the effect of vehicle (0.1% DMSO), GPR35 agonist (TC-G 1001; 10 µM) or GPR35 agonist (TC-G 1001; 10 µM) combining with GPR35 antagonists (ML145; 1 µM) on acrolein (300 µM) induced depolarisation in rat vagus nerve. C) Graph showing effect of vehicle (0.1% DMSO), GPR35 agonist (TC-G 1001; 10 µM) or GPR35 agonist (TC-G 1001; 10 µM) combining with GPR35 antagonists (ML145; 1 µM or CID2745687; 1 µM) on acrolein (300 µM) induced depolarisation in guinea pig vagus nerve. D) Graph showing effect of vehicle (0.1% v/v DMSO), GPR35 agonist (TC-G 1001; 10 µM) or GPR35 agonist (TC-G 1001; 10 µM) combining with GPR35 antagonist (ML145; 1 µM) on acrolein (300 µM) induced depolarisation in human vagus nerve. Data displayed as mean ± S.E.M. percentage inhibition of control responses, n=4. 4 donor patients: 3 males, 36-57 years old and 1 female, 45 years old. *p<0.05 comparing depolarisation levels before and after treatment using a paired t-test

4.3.2 Effect of GPR35 receptor in TRPA1-mediated calcium imaging in isolated vagal ganglia neurons

In 4.3.1, I have successfully shown that the activation of GPR35 inhibits TRPA1-induced depolarisation in the isolated vagus nerve, similar to DSCG in rat, guinea pig and human tissue. As mentioned in 3.3.5, although recording vagus depolarisation is a relatively high-throughput and pharmacologically amenable technique, the vagus nerve innervates not only the respiratory system, but also the heart and other organs. To ensure that the observed effect would play a role on airway specific innervation, intracellular calcium flux was assessed as a marker of activation in airway-specific neurons. Firstly however, single cell PCR was used to determine whether GPR35 are expressed by vagal ganglia airway sensory neurons. As can be seen in Figure 4.6, GPR35 were expressed at the mRNA level in vagal airway sensory neurons.

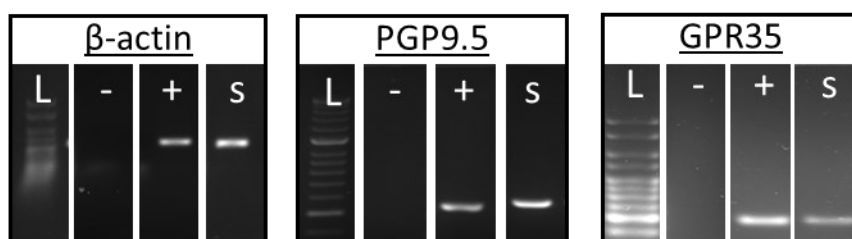


Figure 4.6 Expression of GPR35 in individual vagal airway sensory neurons

Example gel pictures of GPR35 expression. L is ladder; - is water control; + is positive control; s is single cell cDNA. Left panel is the β -actin is a loading control; the middle panel is the neuronal marker PGP9.5; the right panel is the expression of GPR35.

After showing that airway neurons express GPR35, I next wanted to confirm they were functional. Calcium flux was assessed as a marker of neuron activation, as detailed in methods section 2.3.4. The effect of the GPR35 agonist TC-G 1001 on TRPA1-driven neuron activation was determined.

In the presence of TC-G 1001, the calcium influx induced by acrolein was significantly reduced. This is indicated in example images (4.7A), example traces (4.7B) and graphically (4.7C). Therefore, this data suggests that the inhibitory effect of GPR35 agonist TC-G 1001 on TRPA1 channel activation occurs in airway-specific vagal ganglia sensory neurons.

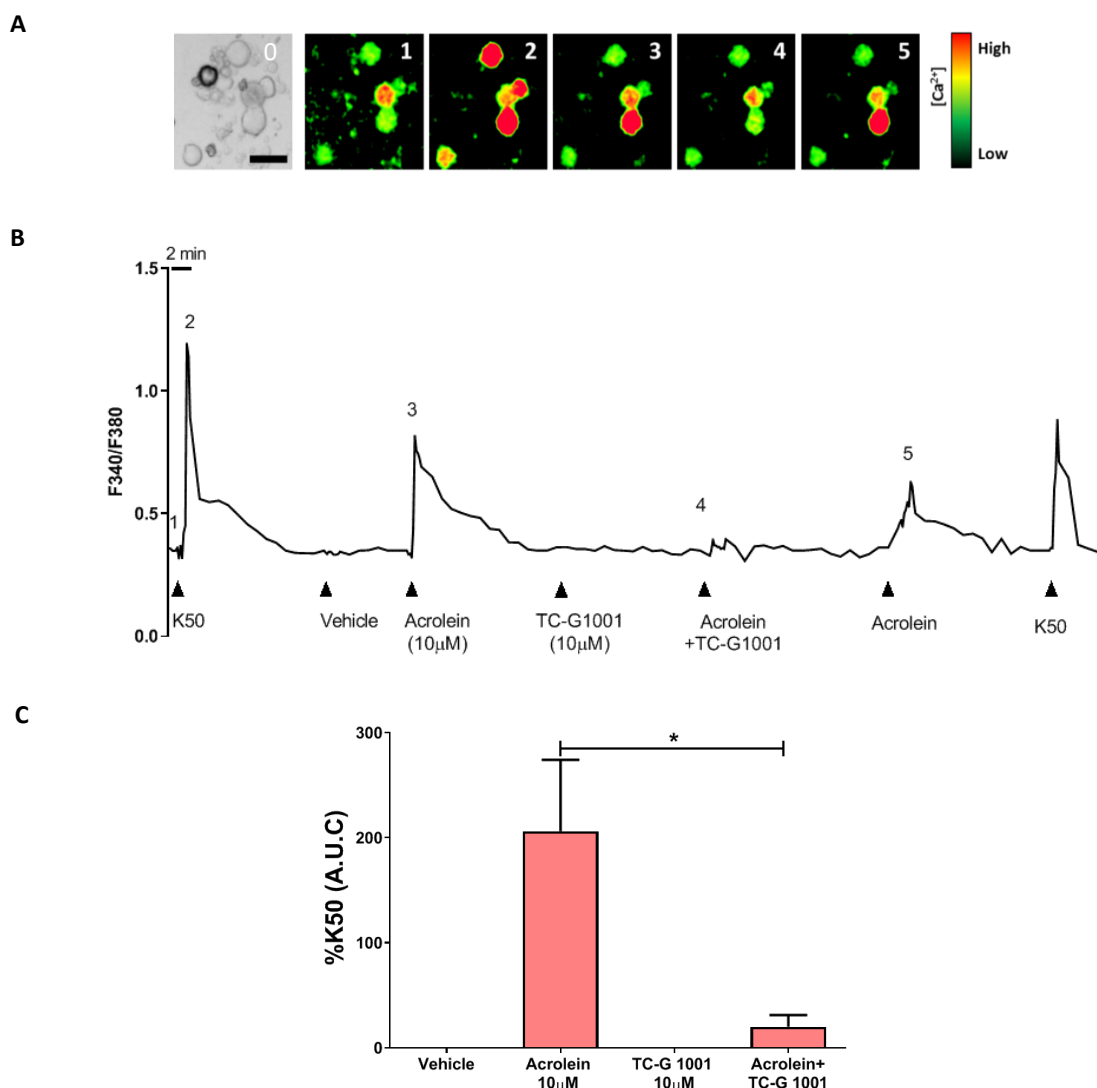


Figure 4.7 Effect of GPR35 agonist TC-G 1001 on TRPA1 induced calcium-flux in guinea pig isolated airway specific neuron

A) Example of bright field and colour coded images of intracellular calcium levels in isolated airway neurons. Increase in red indicates increase of calcium with a colour indicator to the right. Picture number 1 to 5 on images indicates the corresponding point on the example trace below (0: Bright field; 1: Before treatment; 2: High potassium solution K50 (50 mM potassium); 3: Acrolein (10 μ M); 4: Acrolein in presence of GPR35 agonist TC-G1001 (10 μ M); 5: Acrolein). Scale bar is 50 μ m. B) The example trace of intracellular calcium levels induced by drug treatments was determined from analysis of images taken every 10s, and displayed as fluorescence ratio F340/F380 with baseline corrected. C) Summary graph showing the effect of DSCG on acrolein-induced intracellular calcium levels in neurons as determined by calculation of area under curve (AUC) from traces of each experiment. Data are presented as mean $\pm$ S.E.M. $n = 5-19$ neurons harvested from 2 guinea pigs. * $p < 0.05$ comparing calcium levels before and after treatment using a paired t-test.

4.3.3 Effect of GPR35 receptor in DSCG-mediated inhibition of TRPA1-driven depolarisation of isolated vagus nerves

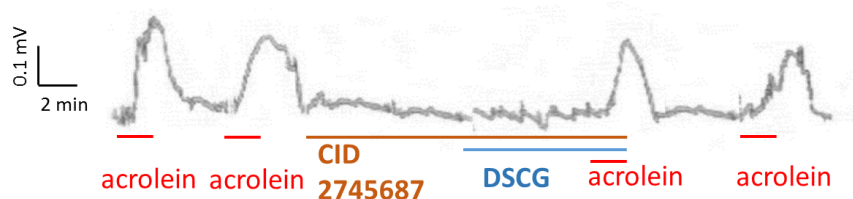
After profiling the effect of GPR35 on TRPA1 agonist acrolein induced sensory nerve activation, the next aim of this chapter was to investigate the role of GPR35 in DSCG-induced inhibition of the sensory

nerve activation. A previous study has shown that DSCG is a potent agonist of GPR35^{191,192}. Therefore by blocking the GPR35 receptor, the inhibitory effect of DSCG should be decreased.

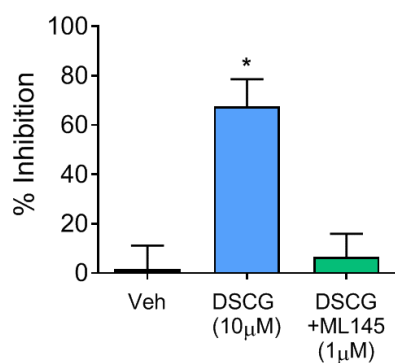
The GPR35 antagonist ML145 was used to investigate that the inhibition induced by DSCG was abolished through antagonising GPR35. As can be seen in Figure 4.8, GPR35 antagonist ML145 (1 μ M) was able to reduce the inhibitory effect of DSCG (10 μ M) in rat, guinea pig and human vagus nerve, by decreasing the inhibition from $67.7 \pm 5.4\%$ to $6.5 \pm 9.5\%$, from $71.6 \pm 6.2\%$ to $24.6 \pm 8.8\%$, from $81.3 \pm 3.7\%$ to $10.3 \pm 3.2\%$ respectively, which suggests that the inhibition of DSCG on TRPA1 agonist acrolein induced depolarisation was via the GPR35 receptor.

A second GPR35 antagonist CID2745687 (1 μ M) was applied for confirmation in guinea pig tissue (Figure 4.8, C). Similarly to ML145, the percentage inhibition was decreased from $71.6 \pm 6.2\%$ to $10.5 \pm 16.6\%$, indicating that GPR35 antagonist CID2745687 (1 μ M) was able to reverse the inhibitory effect of DSCG.

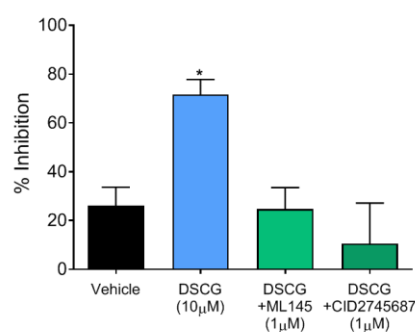
A: Guinea pig



B: Rat



C: Guinea pig



D: Human

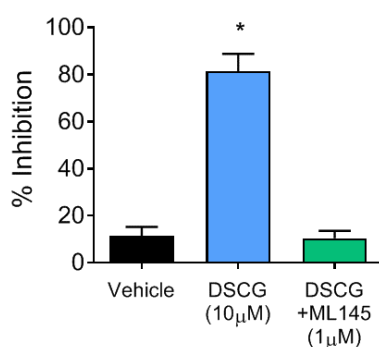


Figure 4.8 Effect of GPR35 antagonists on DSCG-mediated inhibition of TRPA1 driven depolarisation

A) Example trace of GPR35 antagonist CID2745687 (1 μ M) against DSCG-mediated inhibition of TRPA1 agonist acrolein on guinea pig vagus nerve. B) Graph showing effect of vehicle (0.1% v/v DMSO), DSCG (10 μ M) or DSCG combining with GPR35 antagonists (ML145; 1 μ M) on acrolein (300 μ M) induced depolarisation in rat vagus nerve. B) Graph showing effect of vehicle (0.1% v/v DMSO), DSCG (10 μ M) or DSCG combining with GPR35 antagonists (ML145; 1 μ M or CID2745687; 1 μ M) on acrolein (300 μ M) induced depolarisation in guinea pig vagus nerve. C) Graph showing effect of vehicle (0.1% v/v DMSO), DSCG (10 μ M) or DSCG combining with GPR35 antagonists (ML145; 1 μ M) on acrolein (300 μ M) induced depolarisation in human vagus nerve. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, n=4. 4 donor patients: 3 males, 36-57 years old and 1 female, 45 years old. * $p < 0.05$ comparing depolarisation levels before and after treatment using a paired t-test

4.3.4 Effect of GPR35 on DSCG-mediated inhibition in TRPA1-driven single fibre action potential firing

In 4.3.1 and 4.3.3, by using whole vagus nerve tissue *in vitro*, the activation of GPR35 was shown to inhibit TRPA1-driven airway nerve activation, mimicking the inhibitory effect of DSCG demonstrated in chapter 3. Further, GPR35 antagonists blocked the inhibitory effect of GPR35 agonists on sensory nerves. Moreover, GPR35 antagonists also blocked DSCG-mediated inhibition of TRPA1-driven airway nerve activation, which strongly suggests that the inhibitory effects of DSCG on sensory nerve activation is via the activation of GPR35.

Although whole isolated vagus nerve recording is a useful system to pharmacologically assess test nerve activation, this technique studies depolarisation as a proxy for action potential firing in an *in vitro* system. Therefore I used a technique that allows recording of *in vivo* single fibre action potential firing in the guinea pig to translate my *in vitro* findings to a complex *in vivo* system. In chapter 3.3.6 I showed that DSCG inhibited acrolein-evoked firing of C-fibres *in vivo*. Here I evaluated the effect of GPR35 antagonists on DSCG-mediated inhibition of TRPA1-driven action potential firing.

As can be seen in Figure 4.9, acrolein¹⁴⁵ (10mM) successfully triggered firing of guinea pig afferent C-fibres. Following acrolein induced firing, the GPR35 receptor antagonist, ML145 (30mg/kg, i.p.) was administered and the animal was left for 30 minutes. Following that, either DSCG (30 mg/kg, i.t.) or (1% v/v ethanol in saline; i.t.) was administered to the animals, left for 30 min. Subsequent C-fibre activation by acrolein was observed in the ML145/DSCG treated group, while in the vehicle/DSCG group C-fibre activation by acrolein was significantly inhibited. Capsaicin was applied at the start and end of the experiments to help select for C-fibres and to demonstrate viability of the nerve.

This data indicates that the inhibitory effect DSCG on C-fibre firing *in vivo* is blocked by antagonising GPR35.

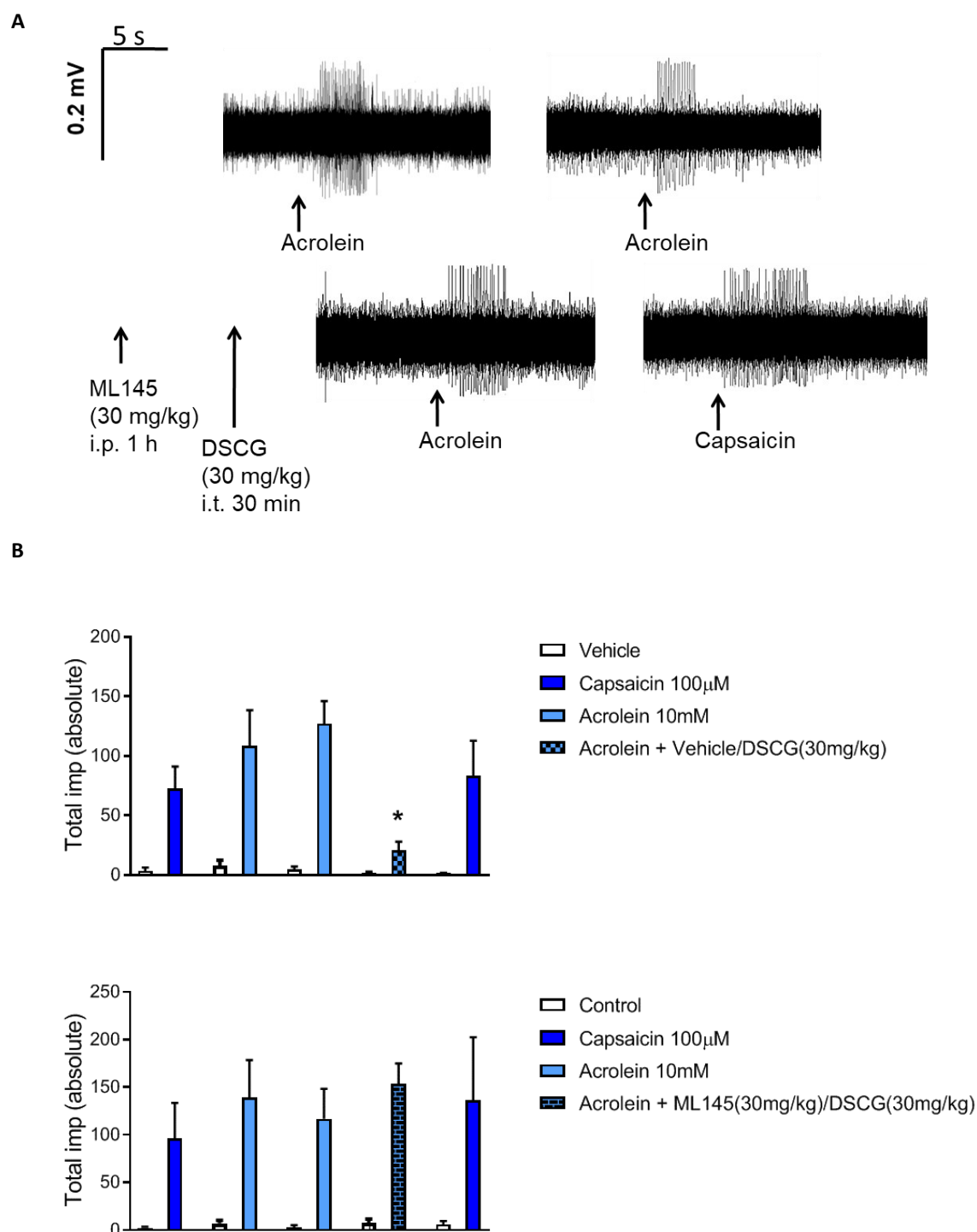


Figure 4.9 Effect of GPR35 antagonist on DSCG-mediated inhibition in TRPA1-driven single fibre action potential firing

A) Example trace of the effect of GPR35 antagonist (ML145 1 µM) on DSCG mediated inhibition in TRPA1-induced single fibre firing. C) Two Panels show the total impulses with and without GPR35 antagonist ML145 application. Data is expressed as mean $\pm$ S.E.M. $n=3$. * $p<0.05$, paired t-test comparing with and without GPR35 antagonist treatment.

4.3.5 Effect of GPR35 on DSCG-mediated inhibition of late asthmatic response (LAR)

As shown in section 3.3.1, DSCG attenuated LAR following OVA challenge in a rat model of allergic asthma. Having shown in this chapter that the effect of DSCG on TRPA1-mediated nerve activation is via GPR35, the final aim of this chapter was to investigate if GPR35 is involved in DSCG attenuation of the LAR. To investigate this, BN rats were dosed with vehicle or the GPR35 antagonist ML145 prior to DSCG administration and OVA challenge.

Figure 4.10 shows average PenH recording over time: Following OVA challenge the LAR response in the in vehicle treated group (Navy blue trace) group significantly increased compared to the saline challenged control group which did not display a response (Black trace). The vehicle treated group LAR response peaked at 2-3 hours and lasted for up to 5 hours. This response was used as reference to assess changes induced by treatments. Treatment with ML145 (1mg/kg) by itself showed no effect on OVA-driven LAR. Treatment with 10 mg/kg DSCG significantly inhibited the OVA-driven LAR, whereas in the ML145/DSCG treated group the inhibitory effect from DSCG was attenuated. These data obtained with the BN rat LAR model demonstrate that GPR35 is required for DSCG-mediated inhibition of LAR bronchospasm.

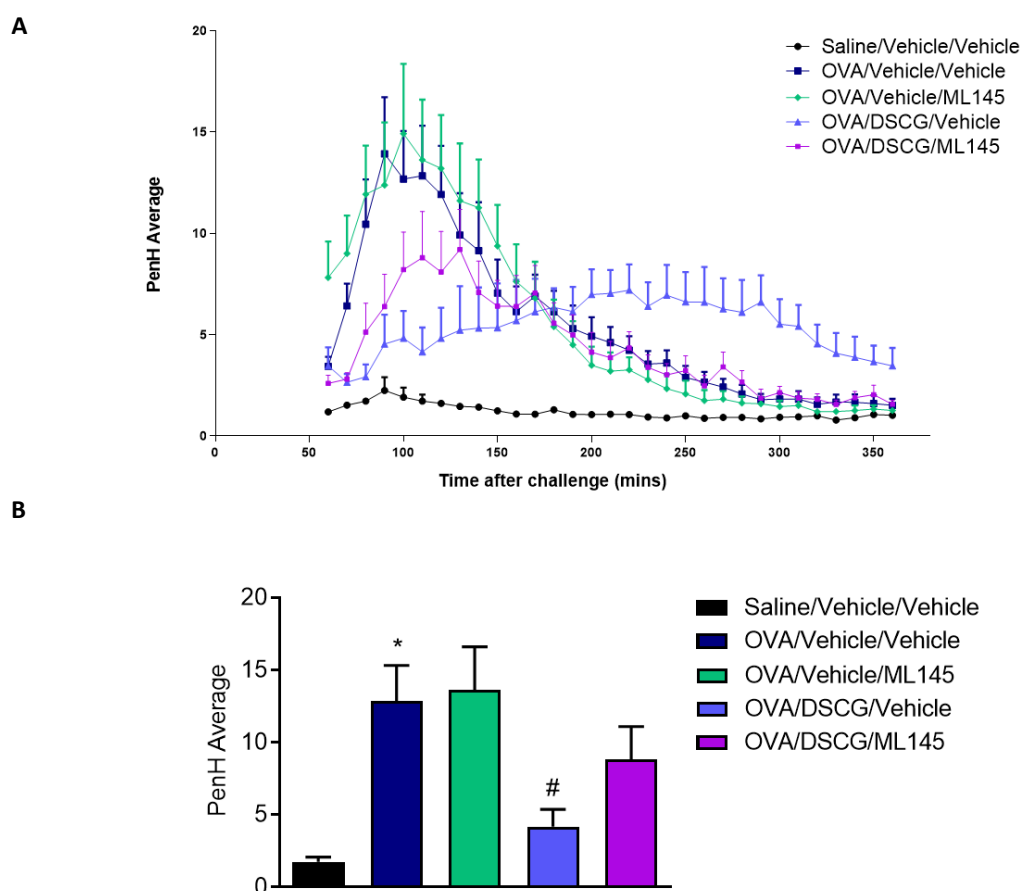


Figure 4.10 Effect of GPR35 antagonist on DSCG-mediated inhibition of OVA-driven LAR

The effect of GPR35 antagonist ML145 on DSCG-mediated inhibition of OVA induced late asthmatic response at dose of 1 mg/kg.

*All groups were sensitised by OVA. OVA/DSCG/ML145 stands for the group challenged by OVA and dosed by ML145 (30 mg/kg) and DSCG (10 mg/kg). OVA/DSCG/Vehicle stands for the group challenged by OVA and dosed by vehicle of ML145 (0.5% methylcellulose + 0.2% tween80 in saline, v/v) and DSCG. OVA/Vehicle/ML145 stands for the group challenged by OVA and dosed by ML145 (30 mg/kg) and vehicle of DSCG (0.5% v/v ethanol in saline). OVA/Vehicle/Vehicle stands for the group challenged by OVA and dosed by both vehicles. Saline/Vehicle/Vehicle stands for the group challenged by Saline and dosed by both vehicles. Data is expressed as (A) Penh average over the recording time period; (B) Penh average at peak response (110 mins). Data expressed as mean $\pm$ S.E.M. n=8. * $p < 0.05$ comparing saline and OVA challenged, vehicle treated groups using a Mann Whitney test. # $p < 0.05$ comparing OVA challenged vehicle dosed group with other drugs dosed group using Kruskal-Wallis with Dunn's multiple comparisons test.*

4.4 Discussion

The aim of this chapter was to investigate the hypothesis that DSCG may activate GPR35 to modulate TRPA1 on airway sensory nerves.

To achieve this, firstly recording of isolated vagus nerve depolarisation *in vitro* was selected as a pharmacologically amenable technique to study the effect of GPR35 on the TRPA1-driven nerve activation. As mentioned previously, DSCG was hypothesised to have its effect via activating GPR35. Therefore a GPR35 agonist TC-G 1001 was selected to assess if it could mimic the inhibitory effect of DSCG²⁴⁷.

Initially, a concentration response of GPR35 agonist TC-G 1001 (1 nM to 100 μ M) against TRPA1 agonist acrolein (300 μ M) induced depolarisation was carried out in rat vagus nerves, where TC-G 1001 caused a concentration dependent inhibition of acrolein induced nerve activation. Two more GPR35 agonists, kynurenic acid and zaprinast, were employed to confirm the role of GPR35 in TRPA1 induced depolarisation. These three GPR35 agonists also inhibited acrolein induced depolarisation in guinea pig and in human isolated vagus nerves, indicating that this effect is not species specific and could also translate to the clinic. This also indicates that GPR35 agonists can mimic the effect of DSCG as previous data showed that DSCG was able to inhibit TRPA1 induced activation of nerves in rat, guinea pig and human isolated vagus nerves.

After investigating the effect of GPR35 agonists on TRPA1 agonist acrolein induced nerve depolarisation, the effect of the GPR35 antagonist ML145 was then evaluated to determine that the inhibition induced by GPR35 agonists at this concentration was GPR35-specific. ML145 was able to reverse the inhibitory effect of GPR35 agonist TC-G 1001 (10 μ M) in rat, guinea pig and human vagus nerve, which suggests that the inhibition of GPR35 agonist TC-G 1001 on TRPA1 agonist acrolein

induced depolarisation was specifically via GPR35. A second GPR35 antagonist CID2745687 was applied for confirmation in the guinea pig and it was able to reverse the inhibitory effect of GPR35 agonist TC-G 1001, similar as ML145.

Whilst the inhibitory effect of GPR35 on TRPA1-mediated nerve activation was demonstrated in isolated whole vagus nerve tissue, it is known that the vagus nerve innervates a number of organs and tissues alongside the lungs²⁴⁰. Therefore, in order to clarify that GPR35 agonist exerts the inhibitory effect on TRPA1 on airway sensory nerves, vagal ganglia neurons specifically innervating the airway were isolated and intracellular calcium levels assessed as a marker of neuron activation. Expression of GPR35 in airway specific vagal neurons was firstly determined by single cell RT-PCR. This technique allows the detection of target expression in individual neurons. Following this, the GPR35 agonist TC-G1001 was utilised to investigate the effect of GPR35 on TRPA1-induced increases in intracellular Ca^{2+} in airway specific neurons. Activation of the neurons by acrolein was inhibited by a GPR35 agonist in guinea pig neurons. These results demonstrated that the inhibitory effect of GPR35 agonist on TRPA1 initiated nerve activation was indeed acting on airway-specific neurons.

By using *in vitro* techniques to evaluate GPR35 ligands, I confirmed that the GPR35 agonist can inhibit TRPA1-driven airway nerve activities and this effect can be reversed by a GPR35 antagonist. The next step was to clarify if DSCG exerts its inhibitory effect via activating GPR35. By applying a GPR35 antagonist before DSCG in vagal nerve recording preparation, the effect of DSCG on TRPA1-driven nerve activation was observed while GPR35 receptor was blocked. Interestingly, GPR35 antagonists blocked the inhibitory effects of DSCG in isolated vagal nerves from rats, guinea pigs and humans. These results demonstrated that the inhibitory effect of DSCG on TRPA1 initiated nerve activation was via the activation of GPR35.

All the *in vitro* techniques described above were very useful to investigate and validate pharmacological tools but did not assess the effect of GPR35 on action potential firing. To circumvent this, *in vivo* single fibre action potential firing recording experiments were carried out to evaluate the effect of GPR35 antagonist ML145 on DSCG-mediated inhibition of TRPA1-driven single afferent fibre firing. By dosing GPR35 antagonist before DSCG, the result showed that GPR35 antagonist can reverse the inhibitory effect of DSCG on TRPA1-induced firing of C-fibre afferents, thus demonstrating that DSCG attenuates TRPA1 driven airway sensory nerve responses via activating GPR35.

Unfortunately, the GPR35 agonists I used in the tissue based systems do not have the appropriate pharmacokinetic profile to be used *in vivo*. So it was not possible to use these tools to confirm the role of GPR35 in this model. In a later chapter I will describe the search and discovery of an appropriate tool compound and its impact in this model system.

The final aim of this chapter was to examine if the impact of DSCG in the model of LAR was via the activation of GPR35. To do so, I determined the effect of a GPR35 receptor antagonist ML145 in the model system. As observed in chapter 3, DSCG again inhibited the LAR; but in the presence of ML145 this blockade was attenuated. Therefore, there is strong evidence to suggest that DSCG attenuates the LAR via acting on GPR35.

Figure 4.11 shows a schematic of this mechanism. The results in this chapter has together suggest that DSCG may activate GPR35 channel to modulate TRPA1 on airway sensory nerves. I can therefore accept my chapter hypothesis.

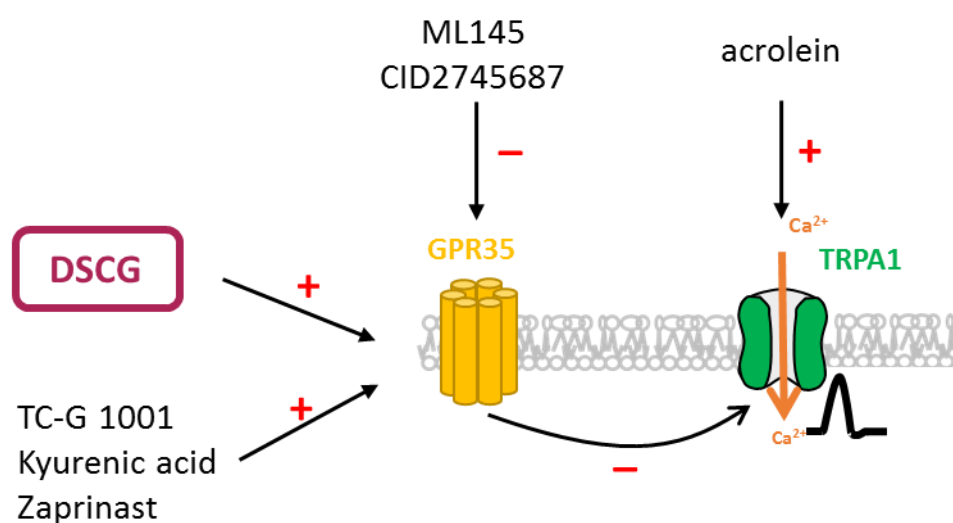


Figure 4.11 Schematic of GPR35 mechanism on TRPA1-induced airway sensory nerve activation

There is little known about the mechanism by which the activation of GPR35 can modulate TRPA1 driven responses in airway sensory innervation. It has been previously reported that activation of GPR35 triggers a Gi dependent inhibition of N-type calcium channels in rat neurons¹⁹⁴. Indeed the role of Gi and N-type calcium channels could be investigated to understand more in terms of the signalling between GPR35 and TRPA1 induced firing of nerves.

Although no endogenous ligands of GPR35 are currently known, the search continues. Indeed, Maravillas-Montero *et al.* suggested that GPR35 could be a receptor for CXCL17 which is known to be constitutively expressed in the lung²⁴⁸. Although others question this claim^{249,23}. Hopefully further research will find endogenous and synthetic ligands for GPR35. There are still huge opportunities to identify agonists, antagonists and signal transduction pathways of GPR35. It may play a critical role in novel signaling pathways and could lead to the identification of new drug targets.

Therefore, my next chapter used a newly developed GPR35 agonist to fully confirm the mechanism I found in this chapter. It investigates if the activation of GPR35 can attenuate TRPA1-driven airway sensory nerve activation and the LAR.

Chapter 5 Confirming the effect of activation of GPR35 with an agonist

5.1 Rationale

In previous chapters, DSCG was shown to inhibit TRPA1-driven stimulation of sensory nerve activation and the LAR via the activation of GPR35. To further substantiate this observation and to confirm the role of GPR35, I wanted to test the impact of a GPR35 agonist. To find a suitable tool a series of compounds we screened by a collaborator and a selective GPR35 agonist, HTL227477, was selected to be characterised.

To achieve this, I firstly tested the effects of HTL227477 on TRPA1-driven sensory nerve activation *in vitro* using the isolated vagus nerve technique. A GPR35 receptor antagonist (ML145) was used to confirm the effect was through GPR35.

Secondly, before moving into *in vivo* studies, a pharmacokinetic (PK) study was carried out to determine the levels and retention of the compound within the lung after intratracheal administration.

Once a suitable dose for *in vivo* administration was determined, the third aim was to determine whether this could inhibit nerve activation. Therefore, the effects of GPR35 agonist HTL227477 were investigated on TRPA1-driven airway afferent C-fibre firing *in vivo*.

The fourth and final aim of this chapter was to investigate if activation of GPR35 by HTL227477, could inhibit the LAR. This was done via the application of HTL227477 to the *in vivo* rat model of OVA driven LAR. By completing these assessments, it would provide strong evidence to confirm the effect of GPR35 on airway sensory nerve activation and the LAR.

5.1.1 Chapter hypothesis

The activation of GPR35 can attenuate TRPA1-driven airway sensory nerve activation and the LAR.

5.1.2 Aims

1. Investigate the effect of the GPR35 agonist HTL227477 in TRPA1-driven stimulation of the sensory nerves *in vitro*;
2. Investigate the PK profile of HTL227477 in the rat;
3. Investigate the effect of GPR35 agonist HTL227477 on TRPA1-driven stimulation of the sensory nerves *in vivo*;
4. Investigate the effect of GPR35 agonist HTL227477 in OVA-driven model of LAR.

5.2 Methods

5.2.1 Isolated vagal nerve recordings

Vagal tissue was dissected from male Brown-Norway rats and placed in the recording set up as previously described in Section 2.6.1. For experiments using the TRPA1 agonist acrolein as the test stimulus, a submaximal dose of acrolein (300 μ M) was used for antagonist experiments.

5.2.1.1 HTL227477 concentration response curves

In order to investigate the effect of GPR35 agonist HTL227477 on TRPA1 driven vagal nerve depolarisation, rat vagal nerve were harvested and mounted in a 'grease gap' recording chamber, as detailed in general methods section 2.6.1, where the nerve was allowed to equilibrate for 10 min prior to experiment. Following this, TRPA1 agonist, acrolein (300 μ M) was perfused and washed twice to obtain 2 similar responses and ensure repeatability. Between stimulations, Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with either vehicle (0.01% v/v DMSO) or HTL227477 (0.001, 0.01, 0.1 or 1 μ M) for 10 minutes and acrolein was then reapplied in the presence of the vehicle or HTL227477. The nerve was then washed and a final application of acrolein was done in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was expressed as percentage of the average of the two initial basal responses.

5.2.1.2 Confirm the effect of HTL227477 is through activation of GPR35

In order to determine if the effect of HTL227477 on TRPA1-driven vagal nerve depolarisation was through GPR35, a selective GPR35 antagonist ML145 was tested to see if it could block the inhibitory effect of a selected sub-maximal concentration of HTL227477. The same protocol as in the previous section was used, except prior to incubation with the GPR35 agonist, the nerve was incubated with vehicle (0.1% v/v DMSO) or the GPR35 inhibitor ML145 (1 μ M) for 10 minutes. Then the GPR35 agonist HTL227477 (1 μ M) was then added to the perfusion for a further 10 minutes and acrolein was then reapplied in the presence of the drugs. As in section 5.2.1.1 a final application of acrolein was done in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial control responses.

5.2.2 Pharmacokinetics study of HTL227477

Male Brown Norway rats were dosed with compound HTL 227477 (0.1 mg/kg, i.t.) and the lung was dissected and snap frozen at 5 different time points: 30 min, 1 h, 2 h, 4 h and 6 h. Frozen lungs were sent to the company Pharmidex (an outsourced contract research organisation) to test concentrations of HTL227477 in rat parenchyma.

5.2.3 In vivo single fibre action potential firing recording

The rat was prepared as described in Section 2.6.2 and as previously described⁷³. Briefly, in order to specifically record airway sensory fibre firing upon stimulation of airways nerve terminals and block any triggered feedback efferent firing, both vagal nerves were cut at the cervical end, and the left vagal nerve used for recording of afferent action potentials. The vagus nerve was dissected down to a single fibre and attached to a platinum electrode where single action potentials could be recorded using Spike 2 data acquisition software via a CED Micro 1401 interface. C-fibres were identified using a set of criteria⁷³ and as described in Section 2.6.2. This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, adaptation Indices, response to capsaicin and citric acid and finally conduction velocity.

After surgery the animals were allowed to stabilise for at least 30 min. Following identification of a lung afferent C-fibre and its sensitivity to capsaicin aerosol (capsaicin 100 μ M aerosol for 15s), the ensuing protocol was pursued: After a control baseline recording of at least 2 min, capsaicin (100 μ M) was administered by aerosol for 15 s and the changes in fibre activity, intra-tracheal pressure and blood pressure were continuously recorded until baseline or a steady state was re-established. 2 control responses were obtained to aerosolised acrolein (15s 10 mM) and after a suitable interval, either HTL227477 (0.1 mg/kg) or (1% v/v ethanol in saline) was administered i.t. to the animals and another response to acrolein obtained, 30 min after administration of HTL227477.

5.2.4 Ovalbumin (OVA)-Driven Rat Model of LAR

The OVA-driven rat model of LAR has been developed in the group previously²⁷. This is a model that mimics the allergic inflammatory response in asthmatics by using ovalbumin protein to sensitise and challenge the animals.

Male Brown Norway rats were sensitised and challenged as described in 2.7.1. On the day of allergen challenge, rats were dosed with vehicle (0.5% v/v ethanol in saline, 1 ml/kg; i.t.) or HTL 227477 (0.1 mg/kg; at a dose volume of 1 ml/kg; i.t.) or DSCG (10 mg/kg) or budesonide (3 mg/kg) 60 minutes before allergen challenge and 30 minutes following the end of allergen challenge. Penh was recorded from 60 minutes after the end of allergen challenge for a period of 5 hours, as detailed in general methods section 2.7.2.2. Right after PenH assessment, rats received a third dose of vehicle, HTL 227477, DSCG or budesonide. Bronchoalveolar lavage (BAL) was collected (2 x 3 ml RPMI, 30 second each) and total/differential leukocyte numbers were assessed. The Penh average was plotted against time following challenge. The Area Under Curve for the various experimental groups was calculated. Students t-test was carried out to compare data between saline and OVA challenged, vehicle treated

groups. One-way ANOVA test was carried out to compare data between OVA challenged vehicle dosed group with HTL227477, DSCG and budesonide treated groups.

5.3 Results

5.3.1 Role of HTL227477 in TRPA1-driven vagal nerve depolarisation

The effect of the selective GPR35 agonist HTL227477 was initially profiled against TRPA1 agonist acrolein induced depolarisation in rat vagus nerves.

A concentration response of GPR35 agonist HTL227477 (1 nM to 1 μ M) against acrolein (300 μ M) induced depolarisation was carried out. The concentration range was selected from previous studies carried out by the collaborative company. As shown on the graph (Figure 5.1), GPR35 agonist HTL227477 inhibited up to $70.2 \pm 5.9\%$ of acrolein driven vagal nerve depolarisation in a concentration-dependent manner, suggesting that HTL227477 is able to attenuate TRPA1 mediated activation of airway sensory nerves. 1 μ M HTL227477 was selected for further investigation as HTL227477 shows a significant inhibitory effect with this concentration.

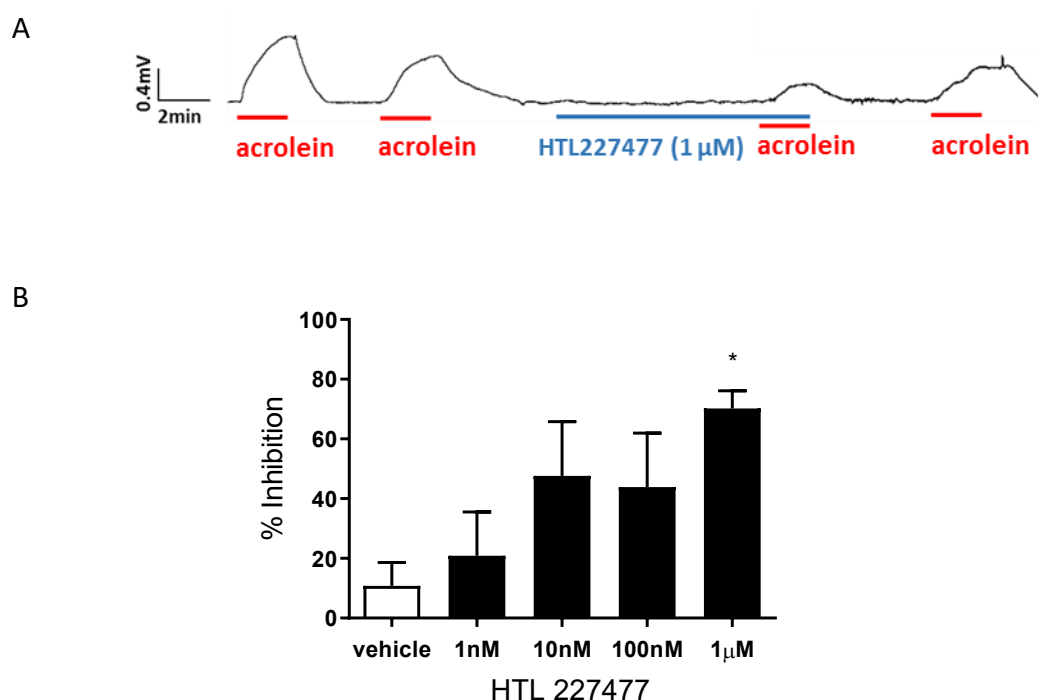


Figure 5.1 Effect of GPR35 agonist HTL227477 on TRPA1 agonist acrolein induced depolarisation in rat

A) Example trace of HTL227477 (1 μ M) on acrolein (300 μ M) induced depolarisation in rat vagus nerve. B) Graph showing effect of vehicle (0.1% v/v DMSO) or HTL227477 (1 nM to 1 μ M) on acrolein (300 μ M) induced depolarisation in rat vagus nerve. $n=4$. Data expressed as mean $\pm$ S.E.M. * $p<0.05$ comparing depolarisation levels before and after treatment using a paired t -test.

After evaluating the effect of HTL227477 on acrolein induced depolarisation, the effect of the GPR35 antagonist ML145 was then evaluated to determine if the inhibition induced by HTL227477 at this dose was GPR35-specific. As can be seen in Figure 5.2, the GPR35 antagonist ML145 (1 μ M) was able to prevent the inhibitory effect of GPR35 agonist HTL227477 (1 μ M) in the rat vagus nerve. This suggests that the new GPR35 agonist HTL227477 inhibited TRPA1 induced depolarisation specifically via GPR35 in the rat.

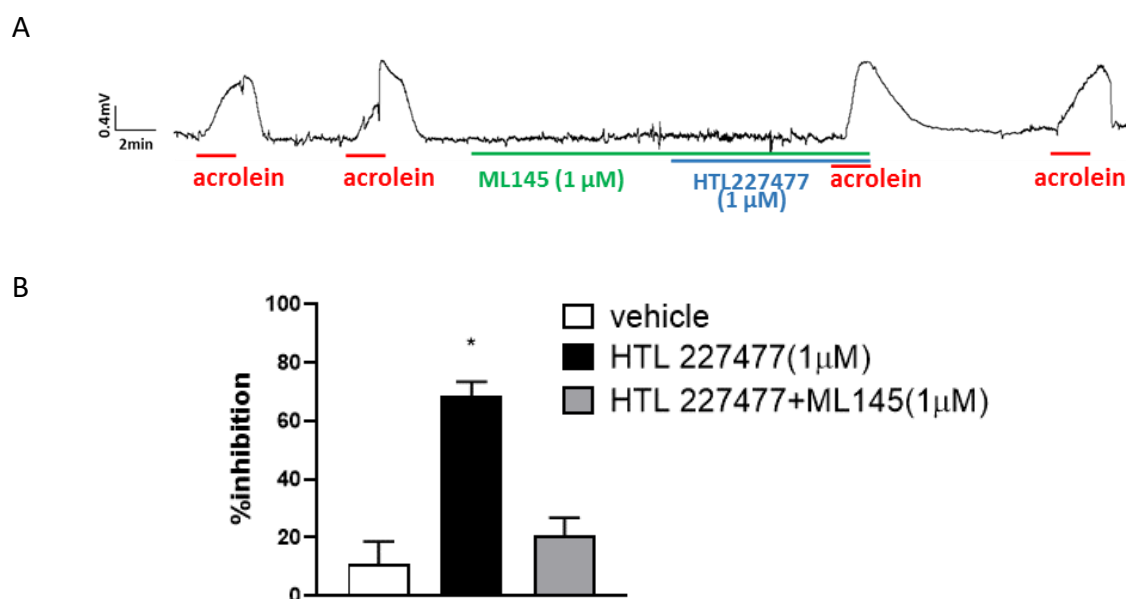


Figure 5.2 Effect of GPR35 antagonist ML145 on HTL227477-mediated inhibition on TRPA1 agonist acrolein induced depolarisation in rat

A) Example trace of GPR35 antagonist ML145 (1 μ M) on HTL227477 (1 μ M)-mediated inhibition of acrolein (300 μ M) induced depolarisation in rat vagus nerve. B) Graph showing GPR35 antagonist ML145 (1 μ M) on HTL227477 (1 μ M)-mediated inhibition of acrolein (300 μ M) induced depolarisation in rat vagus nerve. $n=4$. Data expressed as mean $\pm$ S.E.M. * $p<0.05$ comparing depolarisation levels before and after treatment using a paired t-test.

5.3.2 Pharmacokinetics profile of HTL227477 in rat

In section 5.3.1, I have successfully shown that GPR35 agonist HTL227477 inhibits TRPA1-induced depolarisation in the isolated vagus nerve and this effect can be reversed by GPR35 antagonist ML145, similarly to DSCG. Before being able to use this tool in the *in vivo* systems, I first wanted to determine if it has an appropriate PK profile after intratracheal administration. Time-dependent concentration of HTL227477 in rat lung parenchyma was assessed following administration (0.1 mg/kg, intratracheally). This dose was selected from previous *in vivo* studies carried out by the collaborative company. At each time point after administration, the lungs were excised from the rats. Compound concentration was analysed by Pharmidex and presented by the time after dosing. Concentration of

free HTL227477 was 814.9 ± 191.9 nM, 30 minutes after administration. This concentration was thought to be high enough to activate GPR35 in the *in vivo* model systems.

To confirm the effectiveness of this dose, I utilised the anaesthetised preparation in which airway nerve fibre can be recorded.

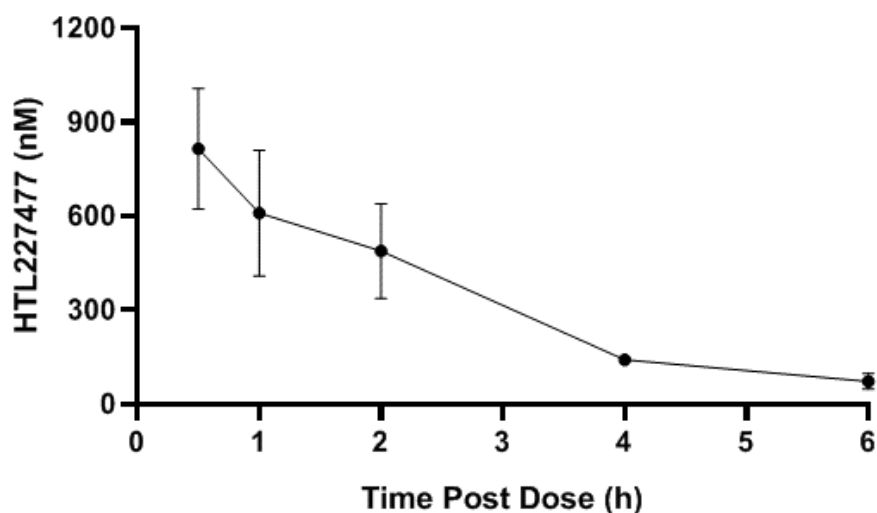


Figure 5.3 Time-dependent concentration of free HTL227477 in rat lung parenchyma

Concentration of HTL227477 in rat lung parenchyma following intratracheal administration (0.1 mg/kg). Data are expressed as mean $\pm$ SEM from 3 animals per time points.

5.3.3 Effects of HTL227477 in TRPA1 driven single fibre action potential firing

As can be seen in Figure 5.4, aerosolised acrolein (10 mM) successfully triggered firing of rat afferent C-fibres. Following acrolein induced firing, HTL227477 (0.1 mg/kg, intratracheally) was administered to the animals. 30 minutes following administration, C-fibre activation by acrolein was successfully inhibited, while in the vehicle group C-fibre activation by acrolein was unchanged. Capsaicin was applied at the start and end of the experiments to help select for C-fibres and to test viability of the nerve fibre. This data indicates that HTL227477 can activate GPR35 at this dose and block acrolein induced firing of C-fibres *in vivo*.

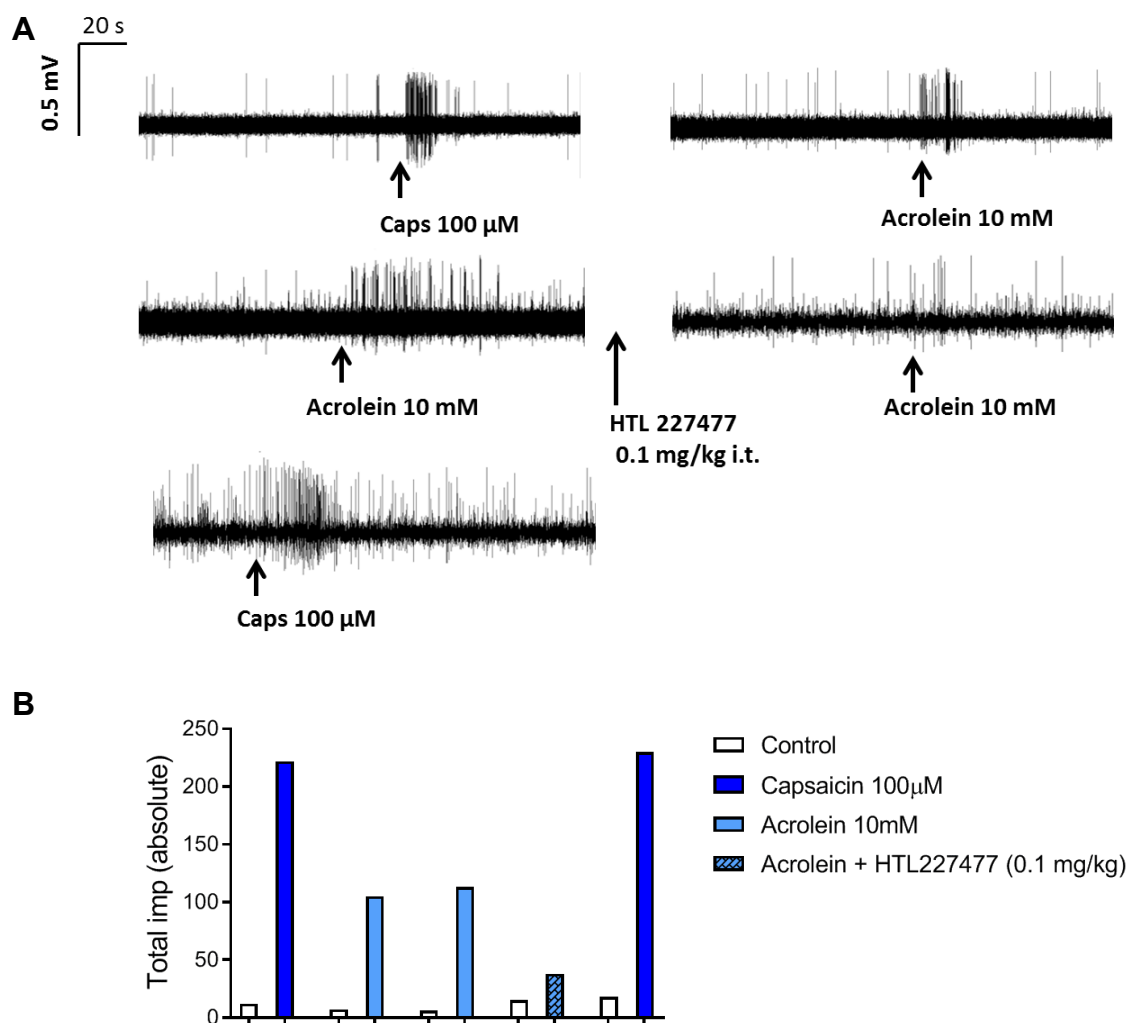


Figure 5.4 Effect of HTL227477 on acrolein (TRPA1) induced firing of C-fibre afferents in rat

A) Example trace of the effect of HTL227477 (0.1mg/kg) on TRPA1 agonist acrolein induced single fibre firing. Starting from top left, each of the panels indicates action potential firing induced by: capsaicin (100 μ M), acrolein (10mM), acrolein (10mM), acrolein with the presence of HTL227477 and capsaicin. B) Graph showing the total impulses before and after HTL227477 application. n=1.

5.3.4 Effects of HTL227477 in ovalbumin (OVA)-driven late asthmatic response (LAR)

Having established that HTL227477 is an agonist of rat GPR35 and that a dose of 0.1 mg/kg dosed intratracheally can block TRPA1 responses *in vivo*, I wanted to use this tool compound to confirm the actions of GPR35 on LAR in the model of allergic asthma. Figure 5.5 A shows average PenH recording over time: following OVA challenge the LAR response in the in vehicle treated group (Navy blue trace) group significantly increased compared to the saline challenged control group which did not display a response (Black trace). In the saline challenged group, treatment with HTL227477 (0.1 mg/kg) showed no effect (light orange trace) which indicated that HTL227477 did not have any impact on normal PenH levels. Treatment with 0.1 mg/kg HTL227477 (orange trace) inhibited the OVA-driven LAR to a similar level as DSCG (light blue trace). The steroid positive control, budesonide, inhibited the LAR as expected.

The Area Under Curve for each experimental group was graphed (Figure 5.5 B). These data obtained with the BN rat LAR model confirms the inhibitory effect of GPR35 on LAR by using HTL227477.

OVA treatment has also been shown to increase the number of inflammatory mediators present in the BAL of challenged animals. In addition to the effect on sensory nerves and the LAR, an anti-inflammatory effect was also observed with HTL227477. The compound reduced the number of eosinophils, neutrophils, lymphocytes and macrophages in the BAL fluid compared with the vehicle-treated group (Figure 5.6). A similar anti-inflammatory effect was seen with DSCG. As a positive control, the steroid budesonide reduced the number of eosinophils, neutrophils, lymphocytes and macrophages in the BAL fluid. This would suggest that HTL227477 could have additional beneficial anti-inflammatory effects, as well as the inhibitory effect on sensory nerves driving the LAR.

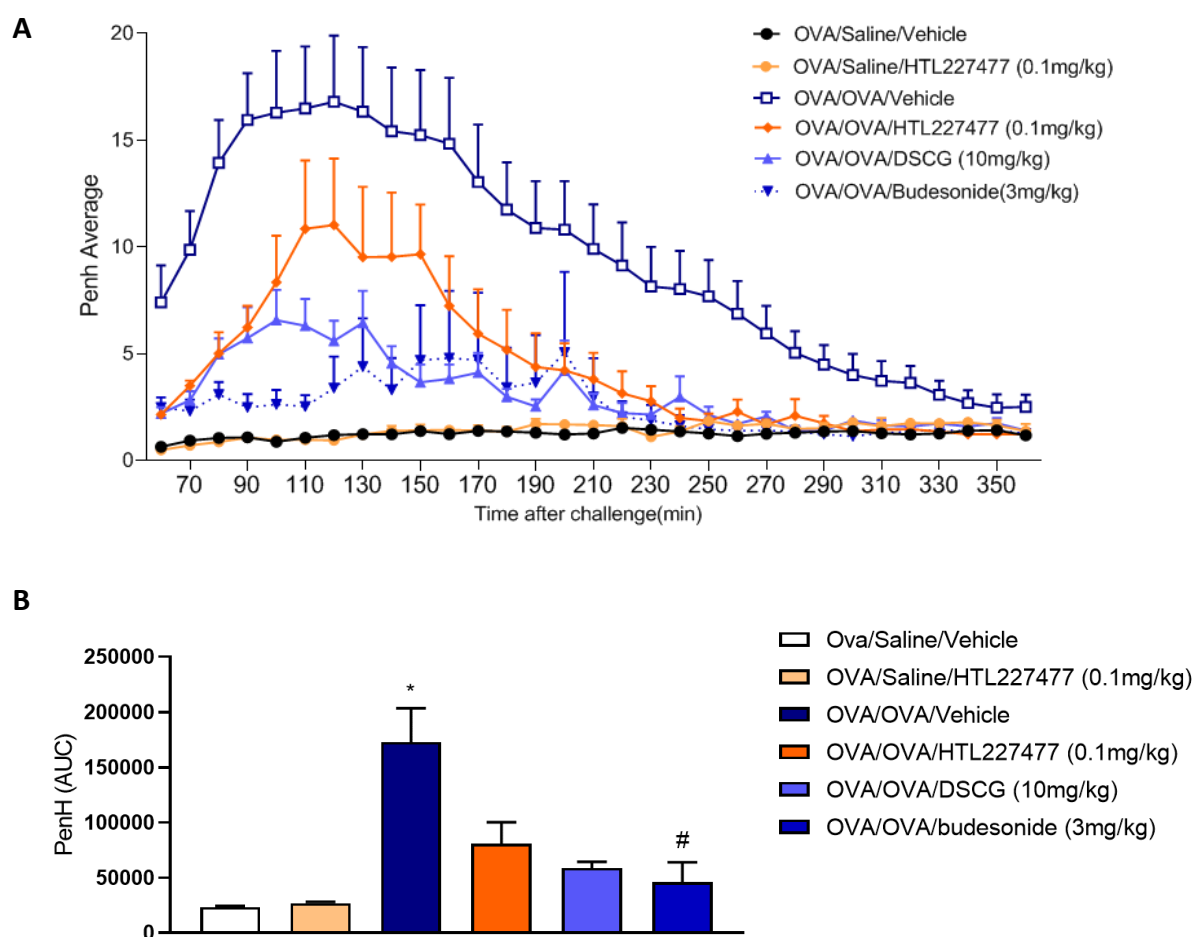


Figure 5.5 Effect of HTL227477 on OVA-driven LAR in rat

The effect of HTL227477 (0.1 mg/kg) on OVA-induced LAR. OVA/Saline/Vehicle stands for the group sensitised by OVA, challenged by Saline and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/Saline/ HTL227477 stands for the group sensitised by OVA, challenged by Saline and dosed by HTL227477 (0.1 mg/kg). OVA/OVA/Vehicle stands for the group sensitised by OVA, challenged by OVA and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/HTL227477 stands for the group sensitised by OVA, challenged by OVA and dosed by HTL227477 (0.1 mg/kg). OVA/OVA/DSCG stands for the group sensitised by OVA, challenged by OVA and dosed by DSCG (10 mg/kg). OVA/OVA/Budesonide stands for the group sensitised by OVA, challenged by OVA and dosed by

budesonide (3 mg/kg). Data is expressed as (A) Penh average (per 10 min) over the recording time period; (B) Total Area Under Curve (AUC) of PenH for each group. Data expressed as mean $\pm$ S.E.M. $n=4-8$. * $p<0.05$ comparing saline and OVA challenged, vehicle treated groups using a Mann Whitney test. # $p<0.05$ comparing OVA challenged vehicle dosed group with HTL227477, DSCG and budesonide treated groups using Kruskal-Wallis with Dunn's multiple comparisons test.

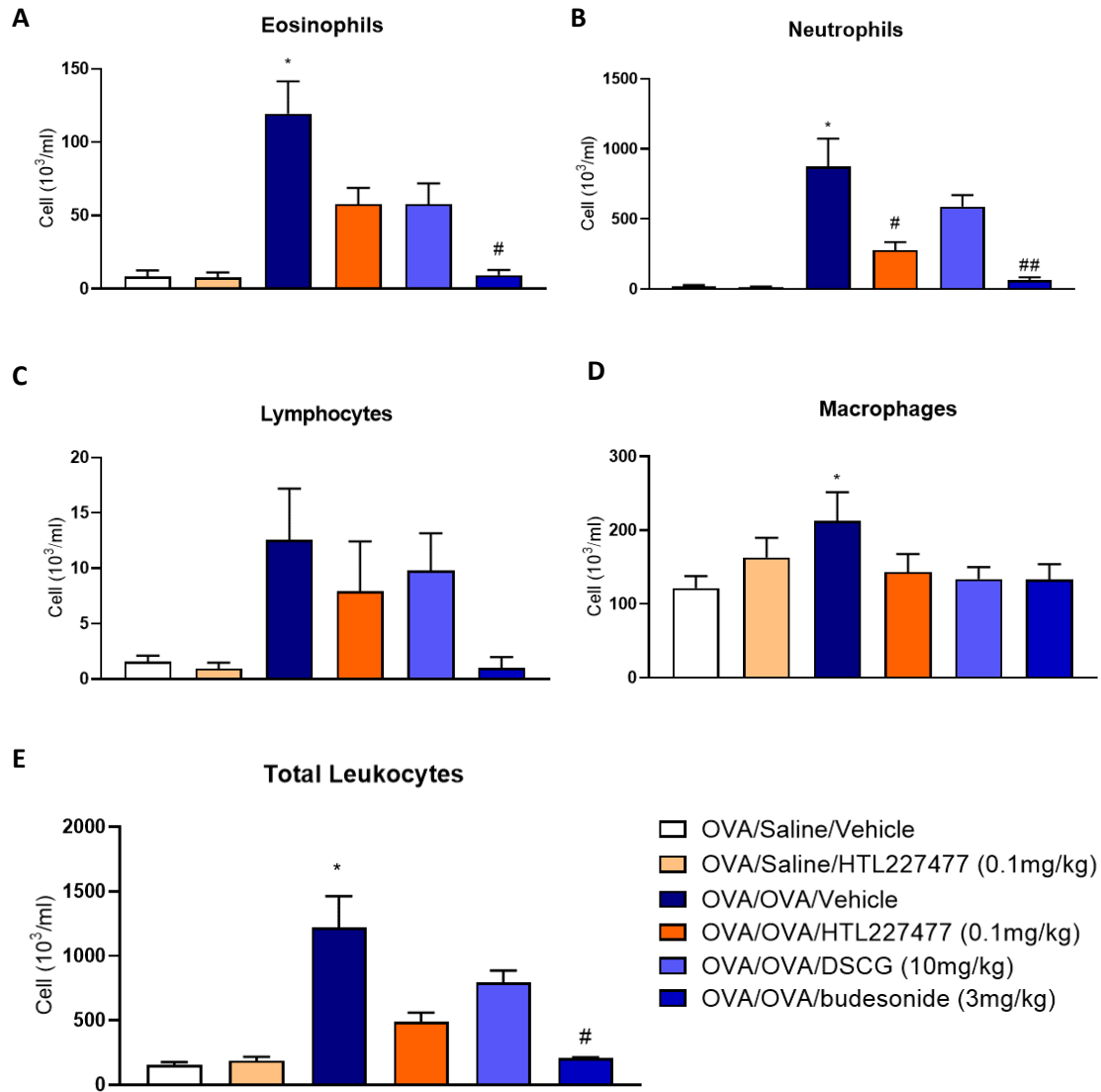


Figure 5.6 The effect of HTL227477 (0.1 mg/kg) on the airway inflammation induced by OVA in the Brown Norway rat.

Data is expressed as cell count in BAL fluid for numbers of: A) Eosinophils; B) Neutrophils; C) Lymphocytes; D) Macrophages and E) Total Leukocytes. OVA/Saline/Vehicle stands for the group sensitised by OVA, challenged by Saline and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/Saline/ HTL227477 stands for the group sensitised by OVA, challenged by Saline and dosed by HTL227477 (0.1 mg/kg). OVA/OVA/Vehicle stands for the group sensitised by OVA, challenged by OVA and dosed by Vehicle (0.5% v/v ethanol in saline). OVA/OVA/HTL227477 stands for the group sensitised by OVA, challenged by OVA and dosed by HTL227477 (0.1 mg/kg). OVA/OVA/DSCG stands for the group sensitised by OVA, challenged by OVA and dosed by DSCG (10 mg/kg). OVA/OVA/Budesonide stands for the group sensitised by OVA, challenged by OVA and dosed by budesonide (3 mg/kg). Data expressed as mean $\pm$ S.E.M. $n=4-8$. * $p<0.05$ comparing saline and OVA challenged, vehicle treated groups using a Mann Whitney test. ## $p<0.01$, # $p<0.05$ comparing OVA challenged vehicle dosed

group with HTL227477, DSCG and budesonide treated groups using Kruskal-Wallis with Dunn's multiple comparisons test.

5.4 Discussion

The aim of this chapter was to use the new GPR35 agonist HTL227477 as a tool to confirm that activation of GPR35 can attenuate the LAR in a rat model of allergic asthma.

To achieve this, firstly recording of isolated vagus nerve depolarisation *in vitro* was used to study the effect of GPR35 agonist HTL227477 on TRPA1-driven nerve activation. A concentration response of the GPR35 agonist HTL227477 (1 nM to 1 μ M) against TRPA1 agonist acrolein (300 μ M) induced depolarisation was carried out in rat vagus nerves. HTL227477 successfully caused a concentration dependent inhibition of the response, indicating that this tool is able to agonist rat GPR35 (our collaborator only had activity data on the human receptor). To confirm the actions of HTL227477 was through the activation of GPR35, I employed a selective antagonist. ML145 was able to block the inhibitory effect of HTL227477 (1 μ M) in rat vagus nerve, which suggests that the activity of HTL227477, like DSCG, was via GPR35.

Having established that this tool compound can activate rat GPR35 and inhibit TRPA1 driven sensory nerve responses, I wanted to use it to confirm my finding with DSCG in the rat model of LAR. Before I could use the tool compound I needed to confirm that its PK profile was appropriate in the rat and that it could block TRPA1 responses. The PK profile after intratracheal dosing was deemed sufficient to activate GPR35 based on the isolated rat vagal data and information from the collaborator. Indeed, when I tested this dose in the anaesthetised rat preparation, it was able to attenuate the TRPA1 triggered firing of the airway nerve. Thus I was confident that I had a tool compound that could activate GPR35 in the rat airway and block TRPA1 stimulated airway sensory nerves.

The final aim of this chapter was to confirm previous findings with DSCG in that activation of GPR35 can attenuate the LAR in a rat model of allergic asthma. These results demonstrated that the GPR35 agonist HTL227477 could inhibit the LAR response. This indicated that activating GPR35 was able to inhibit asthma symptoms in this animal model, similar to what had been seen with DSCG. The results in this chapter has together suggest that the activation of GPR35 was able to attenuate TRPA1-driven airway sensory nerve activation and the LAR. I can therefore accept my chapter hypothesis. Interestingly anti-inflammatory effect was observed with GPR35 agonist HTL227477 which could be other beneficial effect of GPR35 activation. Further investigation could be carried out to understand these effects.

So far all the investigations have been done with the use of TRPA1 agonist acrolein. As reported, TRPA1 is an ion channel which can be activated by a series of stimuli, such as H₂O₂, hyper-osmolarity¹⁴⁸ and diesel exhaust particles¹⁴⁵. In my final chapter I wanted to assess if activation of GPR35 could modulate a range of TRPA1 stimuli.

Chapter 6 Investigation into whether activators of GPR35 and DSCG can modulate responses to a range of TRPA1 triggers

6.1 Rationale

The results of previous chapters have indicated that DSCG is capable of inhibiting TRPA1 on airway sensory nerves through a GPR35 dependent mechanism. The data generated so far has focused on used synthetic ligands such as acrolein and the allergic response in the Brown Norway rat asthma model. TRPA1, however, is also known to be opened by a series of endogenous and exogenous ligands (Figure 6.1), such as prostaglandin E2 (PGE₂), bradykinin (BK), reactive oxygen species, and increases in osmolarity, along with cold temperatures¹⁴⁸. In this chapter, my aim was to determine if DSCG or GPR35 activation can block TRPA1 activation evoked by other mechanisms.

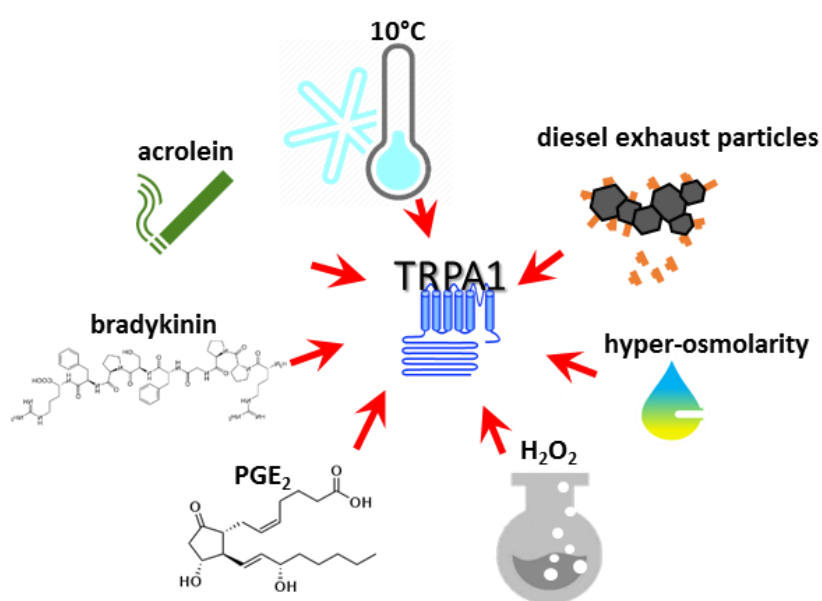


Figure 6.1 TRPA1 activators: acrolein, PGE₂, bradykinin, H₂O₂, hyper-osmolarity, diesel exhaust particles, and 10°C

The initial aim of this chapter was to determine if these reported TRPA1 activators could stimulate the isolated vagal nerve preparation. The next step was to confirm the role of DSCG and GPR35 agonists on these responses.

6.1.1 Chapter hypothesis

DSCG and GPR35 agonist inhibit TRPA1 activation on airway sensory nerves triggered by a range of stimuli.

6.1.2 Aims

1. Investigate the role of TRPA1 in the activation of airway sensory nerves by endogenous and exogenous activators.

2. To investigate the inhibitory role of DSCG and GPR35 agonist in the activation of airway sensory nerves driven by alternate TRPA1 stimuli.

6.2 Methods

6.2.1 Isolated vagal nerve recordings to identify TRPA1 activators

Vagal tissue was dissected from male Brown-Norway rats and Dunkin-Hartley guinea pigs and placed in the recording set up as previously described in Section 2.6.1. Key experiments were repeated with vagal nerve isolated from human tissue to generate translational data.

In order to confirm the selected activators can cause vagal nerve depolarisation and the activation is through TRPA1, a selective TRPA1 antagonist HC-030031 (10 μ M) was employed²⁵⁰. Animal and human tissue were obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following this, reported TRPA1 activators, acrolein (300 μ M), Diesel exhaust particles (DEP-E)¹⁴⁵ (1 μ g/mL), PGE₂¹²⁷ (10 μ M), hyperosmolar solution (+300 mOsm/L), bradykinin⁸⁴ (3 μ M), H₂O₂ (10 mM) or 10°C Krebs solution¹⁴⁸ were perfused for two minutes twice to obtain reproducible responses. Between stimulations, Krebs solution (37°C) was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with either vehicle (0.01% v/v DMSO) or TRPA1 antagonist HC-030031 (10 μ M) for 10 minutes and the same modality was then reapplied in the presence of the antagonist for 2 minutes. The nerve was then washed as previously described and a final application of the same modality applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

6.2.2 Isolated vagal nerve recordings to assess the effect of DSCG and GPR35 agonist on TRPA1-driven response

Guinea pig tissue was obtained, harvested, and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following this, confirmed TRPA1 activators, acrolein (300 μ M), Diesel exhaust particles (DEP-E)¹⁴⁵ (1 μ g/mL), PGE₂¹²⁷ (10 μ M), hyperosmolar solution (+300 mOsm/L), bradykinin⁸⁴ (3 μ M), H₂O₂ (10 mM) or 10°C Krebs solution were perfused for two minutes twice to obtain reproducible responses. Between stimulations, room temperature Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with either DSCG (10 μ M) or GPR35 antagonist TC-G 1001 (10 μ M) for 10 minutes and the same modality was then reapplied in the presence of the drug for 2 minutes. The nerve was then washed as previously described and a final

application of the same modality applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

In order to investigate the effect of GPR35 in DSCG/TC-G 1001 mediated inhibition of TRPA1 driven depolarisation in the isolated vagus nerve, a selective GPR35 antagonist CID2745687 (1 μ M) was applied. Guinea pig tissue was obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. TRPA1 activators, acrolein (300 μ M) or PGE₂ (10 μ M) was perfused and washed twice to obtain 2 similar responses and ensure repeatability. Between stimulations, Krebs solution (37°C) was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with vehicle (0.1% v/v DMSO) or the GPR35 inhibitor CID2745687 (1 μ M) for 10 minutes. Following this the nerve was then incubated with DSCG (10 μ M) or TC-G 1001 (1 μ M) for 10 minutes in presence of the vehicle or CID2745687. Same modality was then reapplied in the presence of the drugs. The nerve was then washed off and same modality was applied again in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

6.2.3 Intracellular calcium signal imaging on isolated vagal ganglia neurons

The cells were loaded with 6 μ M Fura-2 acetoxymethyl ester (Fura-2 AM) at room temperature in the dark for 40 minutes and de-esterified for 30 min. Airway neurons were identified by the presence of Dil fluorescence. Viability of the cells were assessed by applying 50mM potassium solution (K50) at the start and end of experiments.

To study the role of DSCG and GPR35 in the TRPA1 modality PGE₂-driven stimulation pathway, a concentration of 10 μ M PGE₂ was selected based on previous data obtained on the vagal nerve. PGE₂ was applied twice for 30 seconds and washed until the calcium level returned to baseline. Vehicle (0.1% v/v dH₂O in ECS), DSCG (10 μ M) or TC-G 1001 (GPR35 agonist, 10 μ M) was applied for 10 minutes at the end of which PGE₂ was re-applied in presence of the drugs. After a wash off period, PGE₂ was reapplied to assess recovery of the response. Cell viability was assessed by a final response to K50. All recordings were performed at 37°C. Intracellular calcium change was recorded and analysed using HClmage and Image J (As described in chapter 2.3.3). Statistical difference was assessed on PGE₂-induced responses using a paired t-test comparing responses obtained with and without treatment.

6.2.4 Intracellular oxidative stress imaging on isolated vagal ganglia neurons

Airway sensory neurons were harvested and primary cultured at 37°C with 5% CO₂ for 24h in fluorodishes (See chapter 2.3.2). The oxidative responses of isolated vagal ganglia neurons after exposing to stimuli were detected using the superoxide sensitive fluorescent dye dihydroethidium (DHE). Signals were recorded with the same functional imaging system as the one used in calcium imaging experiments as described in 2.3.3. The neurons were loaded with 6 µM DHE for 30 min at 37°C in the dark then washed 3 times for 5 minutes with ECS. All DHE containing solutions, fluorodishes and neurons exposed to DHE were collected at the end of the experiment and sealed in a container for proper disposal by the University.

To study the role of DSCG and GPR35 agonist TC-G 1001 in the PGE₂-TRPA1 pathway induced oxidative stress, a concentration of 10 µM PGE₂ was selected based on the best signals we got using vagus electrophysiological studies and calcium imaging on neurons. Antioxidant N-acetylcysteine (NAC) was used as a positive control.

During the experiments, the drugs were applied as following orders: 1) PGE₂ (10 µM); 2) Drugs (10 µM DSCG, 10 µM TC-G 1001, 10 µM NAC or respective vehicle for drugs/antioxidant (ECS for DSCG and NAC, 0.1% v/v DMSO for TC-G 1001); 3) PGE₂ along with the corresponding antagonist/antioxidant/vehicle; 4) the vehicle for PGE₂ (0.1% v/v ethanol) to ensure the oxidative signal seen with the agonist is not due to the vehicle and 5) PGE₂ alone again.

PGE₂ was applied for 3 minutes and cells were incubated for 10 minutes with the drugs/antioxidants. Images were recorded at a frequency of 0.1 Hertz (1 image every 10 sec). Before each injection, 10 frames were recorded as baseline. After each stimulus application, neurons were carefully washed with ECS six times.

The recordings were analysed using Fiji software²⁰². The generation of superoxide was measured as the peak of the signal minus the baseline recorded prior to adding the drug and data were expressed as mean ± SEM. Statistical difference was assessed on PGE₂-induced responses using a paired t-test between control response and response after treatment with the drug tested.

6.2.5 Isolated vagal nerve recordings to assess the effect of antioxidants on TRPA1 activators-driven depolarisation

In order to assess the effect of antioxidants on TRPA1 modality PGE₂ driven depolarisation, guinea pig and rat tissue were obtained, harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following this, confirmed TRPA1 modality, PGE₂¹²⁷ (10 µM) was perfused for two minutes twice to obtain

reproducible responses. Between stimulations, room temperature Krebs solution was used to wash off the compound and return nerve to baseline state. A third stimulation might be needed if reproducibility was not achieved. The nerve was then incubated with either NAC²⁵¹ (10 μ M) or VAS2870²²⁹ (5 μ M) or DSCG (10 μ M) for 10 minutes and PGE₂ was then reapplied in the presence of the antagonist for 2 minutes. The nerve was then washed as previously described and a final PGE₂ applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

In order to test this inhibition is not via the TRPV1 channel, TRPV1 agonist capsaicin and TRPV1 antagonist were applied. PGE₂¹²⁷ (10 μ M) were perfused for two minutes twice to obtain reproducible responses. The nerve was then incubated with VAS2870 in combination of XEN-D0501²⁰⁹ (10 nM) for 10 minutes and PGE₂ was then reapplied in the presence of the antagonist for 2 minutes. The nerve was then washed as previously described and a final PGE₂ applied in order to test nerve recovery and viability. In the meanwhile, the effect of VAS2870 was tested on responses to capsaicin (1 μ M) following same protocol as above. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

6.2.6 Isolated vagal nerve recordings to assess the effect of TRPV2 antagonists on TRPA1 activators-driven depolarisation

In order to assess the effect of a TRPV2 antagonist on the TRPA1 dependent portion of the PGE₂ induced depolarisation, guinea pig vagus was harvested and mounted in a 'grease gap' recording chamber as detailed in general methods section 2.6.1. The nerve was allowed to equilibrate in the chamber. Following confirmation of the TRPA-1 dependent PGE₂ response, PGE₂¹²⁷ (10 μ M) was perfused for two minutes twice to obtain reproducible responses. Between stimulations, room temperature Krebs solution was used to wash off the compound and return nerve to baseline state. The nerve was then incubated with a TRPV2 antagonist either tranilast^{252,215} (10 μ M) or SKF96365²⁵³ (10 μ M) or NK-4²²⁰ (30 μ M) for 10 minutes and PGE₂ was then reapplied in the presence of the antagonist for 2 minutes. The nerve was then washed as previously described and a final PGE₂ applied in order to test nerve recovery and viability. A change in the level of depolarisation as a result of drug application was reported as percentage of the average of the two initial basal responses.

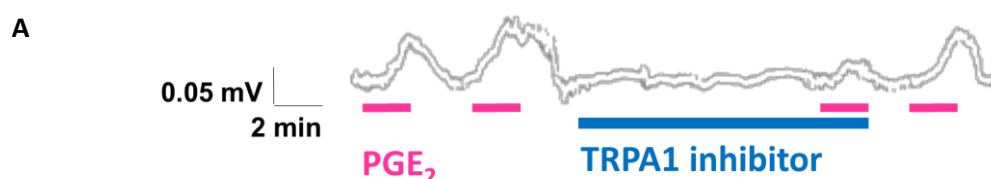
6.3 Results

6.3.1 Characterisation of TRPA1 activators

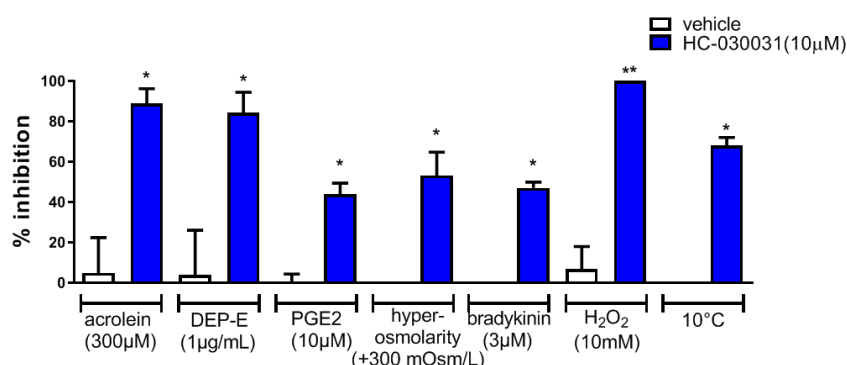
In order to assess if GPR35 agonists exhibited the same inhibitory effects on responses to a range of TRPA1 activators, they were initially characterised against the TRPA1 antagonist HC-030031 (10 μ M) in the isolated vagal nerve recording system.

A range of activators reported to activate TRPA1 (DEP-E, PGE₂, hyperosmolar solution, bradykinin, H₂O₂ and 10°C) were profiled. As shown in Figure 6.2 B, HC-030031 caused significant inhibition of DEP-E, PGE₂, hyperosmolar solution, bradykinin, H₂O₂ and 10°C induced depolarisation. Acrolein was used as positive control. DEP-E and hyper-osmolarity were also profiled in rat tissue, where similarly to the GP, HC-030031 significantly inhibited activation (Figure 6.2 C). In addition, HC-030031 also inhibited PGE₂, DEP-E and bradykinin caused depolarisation in human tissue (Figure 6.2 D). This data therefore suggests that depolarisation by these agents can, at least in part, be attributed to the activation of TRPA1.

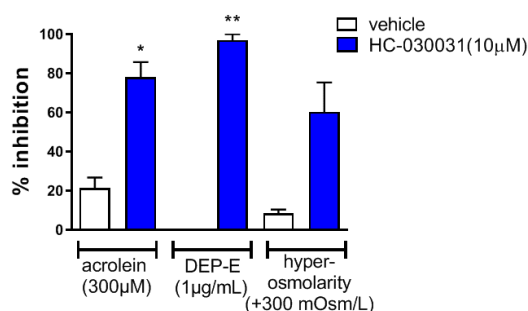
In parallel studies, I showed that the TRPA1 antagonist, at the concentrations used here did not impact on sensory nerve activation triggered by non-TRPA1 stimuli such as TRPV1 and TRPV4 (data not shown).



B: Guinea pig



C: Rat



D: Human

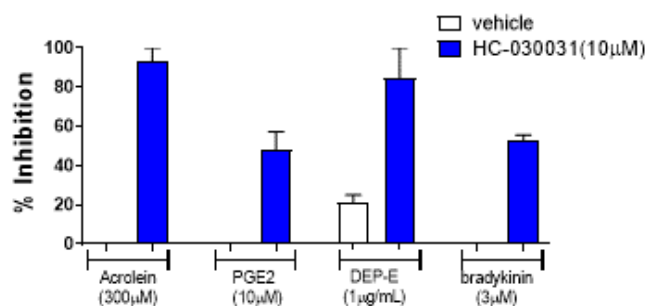


Figure 6.2 Effect of TRPA1 antagonist on depolarisation of guinea pig, rat and human vagus nerve

A) Example trace of TRPA1 antagonist (10 μM) against TRPA1 activator PGE₂ on GP vagus nerve. B) Graph showing effect of TRPA1 antagonist HC-030031 (10 μM) on acrolein (300 μM), DEP-E (1 μg/mL), PGE₂ (10 μM), hyperosmolar solution (+300 mOsm/L), bradykinin (3 μM), H₂O₂ (10 mM) or 10°C induced depolarisation in GP vagus nerve. n=4. C) Graph showing effect of HC-030031 (10 μM) on acrolein (300 μM), DEP-E (1 μg/mL), hyperosmolar solution (+300 mOsm/L) induced depolarisation in rat vagus nerve. n=3-4. D) Graph showing effect of HC-030031 (10 μM) on acrolein (300 μM), PGE₂ (10 μM), DEP-E (1 μg/mL), bradykinin (3 μM) induced depolarisation in human vagus nerve. n=1-2. 2 donor patients: 2 males, 48-56 years old. Data displayed as mean ± S.E.M. percentage inhibition. *p<0.05 **p<0.01 comparing depolarisation levels before and after treatment using a paired t-test.

6.3.2 DSCG but not TC-G 1001 (GPR35 agonist) inhibits vagal nerve activation driven by other TRPA1 activators

Having confirmed a role of TRPA1 in the depolarisation induced by a range of stimuli, I was ready to profile the impact of both DSCG and a GPR35 agonist TC-G 1001. DSCG significantly inhibited the depolarisation caused by acrolein (300 μM), DEP-E (1 μg/mL), PGE₂ (10 μM), hyperosmolar solution (+300 mOsm/L), bradykinin (3 μM), H₂O₂ (10 mM). However, the GPR35 agonist TC-G 1001 inhibited acrolein (300 μM) and DEP-E (1 μg/mL) induced depolarisation but not PGE₂ (10 μM), hyperosmolar solution (+300 mOsm/L), bradykinin (3 μM) or H₂O₂ (10 mM). Neither DSCG nor TC-G 1001 inhibited 10°C induced depolarisation.

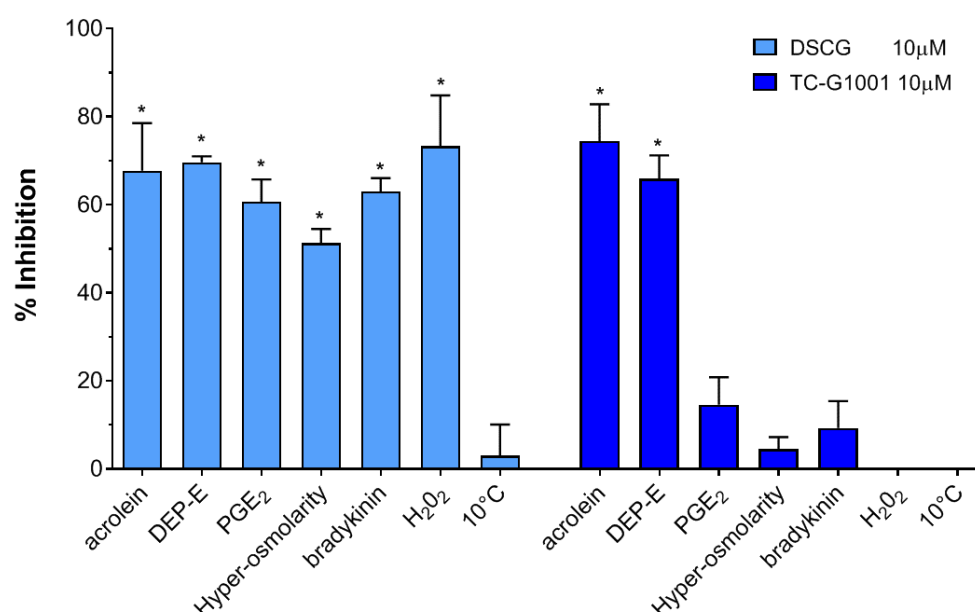


Figure 6.3 Effect of DSCG and GPR35 agonist TC-G 1001 on TRPA1 activators-driven depolarisation of guinea pig

Graph showing effect of DSCG (10 µM) and GPR35 agonist TC-G 1001 (10 µM) on acrolein (300 M), DEP-E (1 µg/mL), PGE₂ (10 µM), hyperosmolar solution (+300 mOsm/L), bradykinin (3 µM), H₂O₂ (10 mM) or 10°C induced depolarisation in GP vagus nerve. n=4. Data displayed as mean ± S.E.M. percentage inhibition. *p<0.05 comparing depolarisation levels before and after treatment using a paired t-test.

To confirm whether the inhibitory effects of DSCG on these alternate TRPA1 modalities was through activation of GPR35, a selective GPR35 antagonist CID2745687 was utilised.

As can be seen in figure 6.4 A and B, GPR35 antagonist CID2745687 (1 µM) was able to reduce the inhibitory effect of DSCG and GPR35 agonist TC-G 1001 on acrolein driven responses, as previously shown. CID2745687 alone did not impact on the vagal system (data not shown). In Figure 6.4 C, however, the GPR35 antagonist was unable to reverse the inhibitory effect of DSCG on PGE₂ driven depolarisation. This suggests that DSCG can inhibit PGE₂-induced depolarisation via a non-GPR35 mechanism.

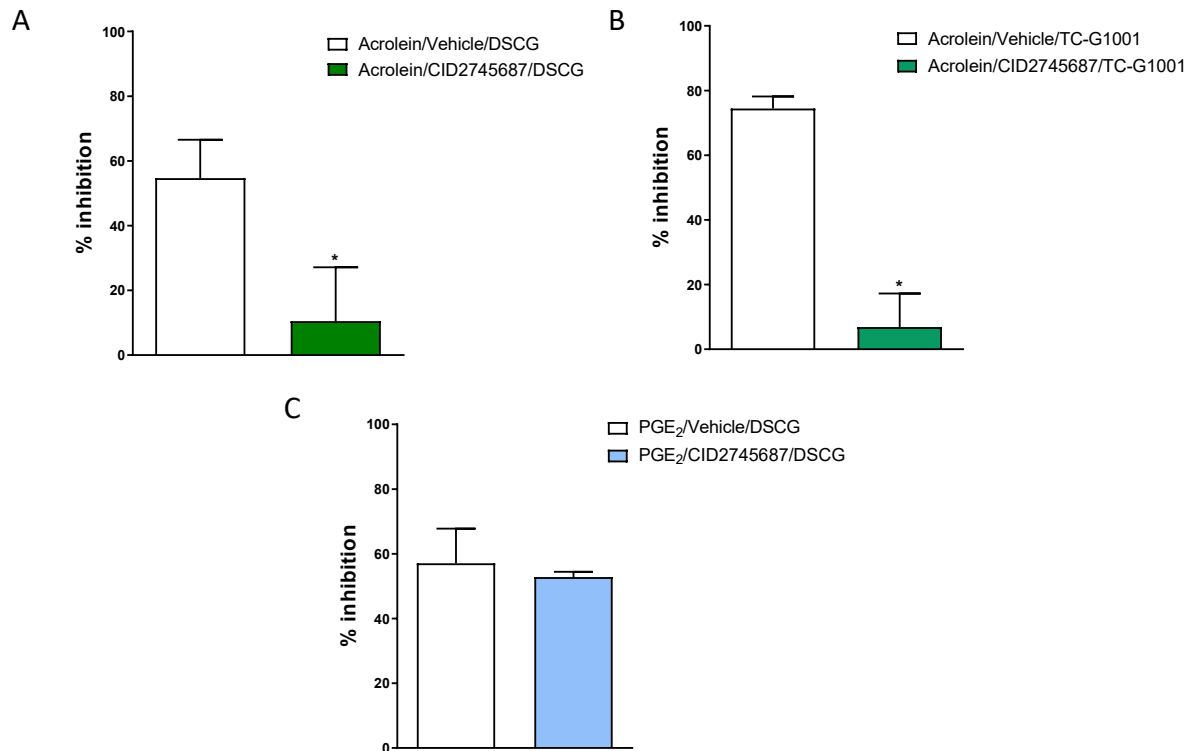


Figure 6.4 Effect of GPR35 antagonists on DSCG/GPR35 agonist mediated inhibition of TRPA1 driven depolarisation

A) Graph showing effect of DSCG (10 μ M) combining with vehicle (0.1% v/v DMSO) or GPR35 antagonists CID2745687 (1 μ M) on acrolein (300 μ M) induced depolarisation in GP vagus nerve. B) Graph showing effect of GPR35 agonist TC-G1001 (10 μ M) combining with vehicle (0.1% v/v DMSO) or GPR35 antagonists CID2745687 (1 μ M) on acrolein (300 μ M) induced depolarisation in GP vagus nerve. C) Graph showing effect of DSCG (10 μ M) combining with vehicle (0.1% v/v DMSO) or GPR35 antagonists CID2745687 (1 μ M) on PGE₂ (10 μ M) induced depolarisation in GP vagus nerve. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, $n=4$. * $p<0.05$ comparing percentage inhibition between different treatments using Mann Whitney test

6.3.3 DSCG but not TCG-1001 (GPR35 agonist) inhibits PGE₂-mediated calcium response in airway sensory neurons

In order to confirm the observation on the vagus, the effect of DSCG and TCG-1001 was tested in airway specific sensory neurons. As illustrated in Figure 6.5 (A-C), PGE₂ elicited a calcium response in airway sensory neurons (Potassium (K50 mM) was used as a reference and to demonstrate cell viability after treatment). The average responses of the whole population of cells are summarised in the bar graphs on the right side of figure 6.5 (D-F). The average response in presence of DMSO was not significantly different from the control (Figure 6.5 A and D). When the cells were exposed to DSCG, the calcium signal elicited by PGE₂ was inhibited (Figure 6.5 B) with the average response significantly decreased by 68% (Figure 6.5 E). As with the vagal preparation, the GPR35 agonist, TC-G 1001, failed to modulate the response to PGE₂ (Figure 6.5 C).

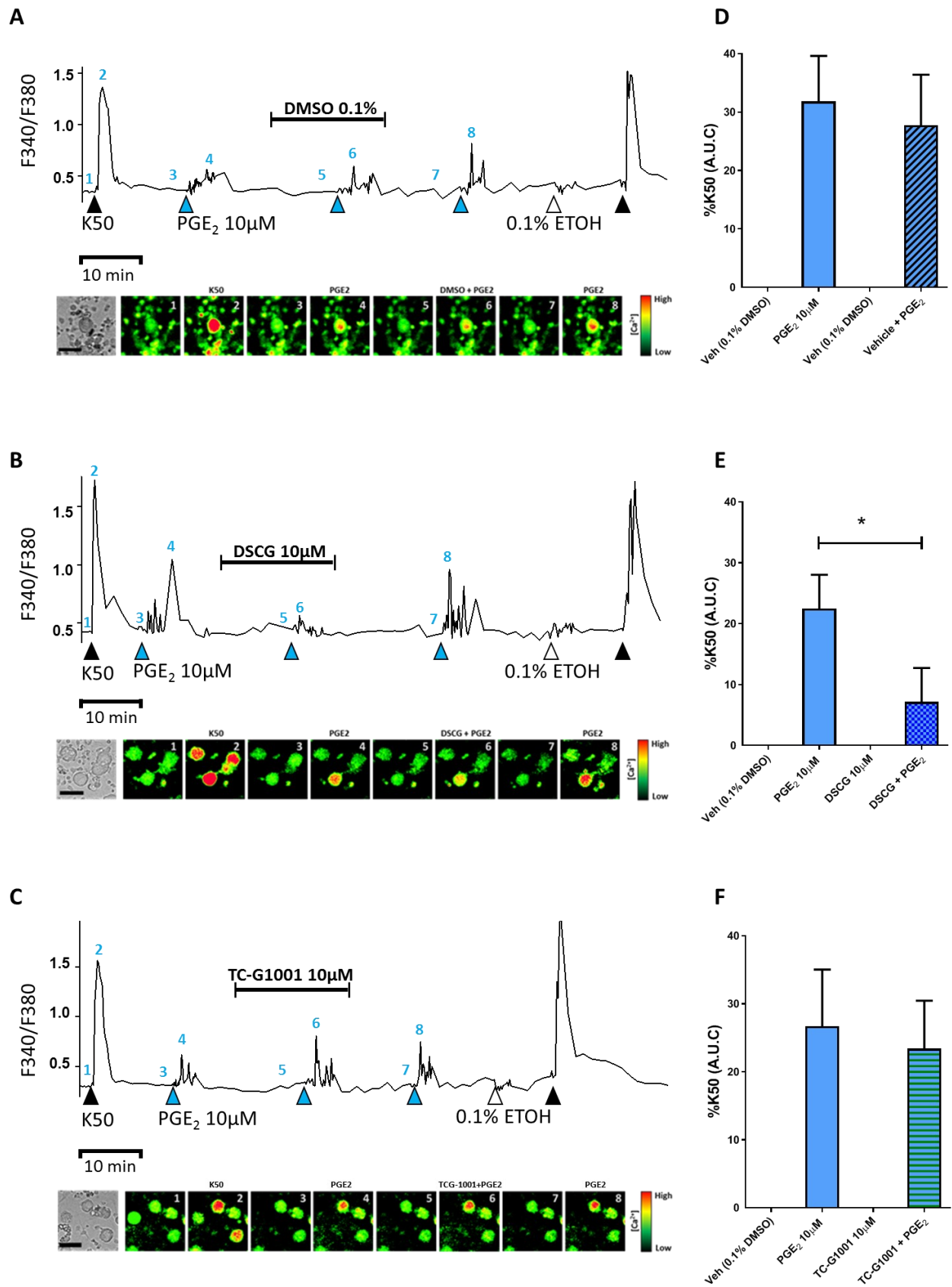


Figure 6.5 Effect of DSCG/GPR35 agonist on TRPA1 (PGE₂)-induced calcium-flux in guinea pig isolated airway specific neurons

A-C) Example traces of intracellular calcium levels recorded over time and expressed as ratio of the light intensity recorded after excitation of the sensor with light wavelengths at 340 nm and 380 nm (F340/F380). Cells were exposed to a 50mM potassium (substituted for sodium) solution (K50), 10 μ M PGE₂ and 0.1% v/v ethanol. Cells used for these recordings are illustrated under the traces. The left image shows the cells in bright field mode with a 100- μ m scale bar. The images labelled 1 to 8 show the ratio of light emitted at 510 nm after successive stimulations at 340 nm and 380 nm corresponding to the time points labelled 1 to 8 in the trace above. D-F) Summary graph showing the effect of PGE₂ on average intracellular calcium signal in neurons for the 0.1% (v/v) DMSO, DSCG and TC-G 1001 treated groups. The calcium response was calculated by measuring the area under curve (AUC) of the responses and expressed as a percentage of the response recorded for the initial exposure to K50. Data are presented as mean $\pm$ S.E.M. * indicate $p < 0.05$ when tested with a paired t-test between test and control response. 13-24 primary airway neurons were harvested from 2-3 guinea pigs and used in this work.

6.3.4 DSCG but not TCG-1001 (GPR35 agonist) inhibits TRPA1 (PGE₂)-mediated oxidative stress response in airway sensory neuron

The clear difference between the impact of DSCG and GPR35 agonist suggested that DSCG had an additional mechanism by which it could modulate TRPA1 responses. TRPA1 is known to be an oxidative stress sensor and respond to reactive oxygen species²⁵⁴. Previously DSCG has been reported as an anti-oxidant¹⁹⁸. Therefore I hypothesised that the inhibition of these alternate TRPA1 modalities could be due to the anti-oxidant properties of DSCG. In order to investigate this, I first wanted to determine if PGE₂ could induce oxidant stress (OS) in the systems I employed using the OS stain Dihydroethidine (DHE). Figure 6.6 shows the oxidative stress responses induced by PGE₂ (10 μ M) compared to its vehicle (ethanol). To confirm this response was OS, I compared the responses with and without an antioxidant N-Acetyl cysteine (NAC) and showed a greatly reduced signal (Figure 6.6).

Once confirmed that PGE₂ induces an oxidative response into the neurons, the effect of DSCG and GPR35 agonist TC-G 1001 on this oxidative stress was investigated. When the neurons were incubated for 10 minutes with DSCG (10 μ M) prior to exposing them to PGE₂, the level of ROS observed was significantly reduced (Figure 6.7 A-B). However GPR35 agonist TC-G 1001 did not reduce the oxidative response (Figure 6.7 C-D).

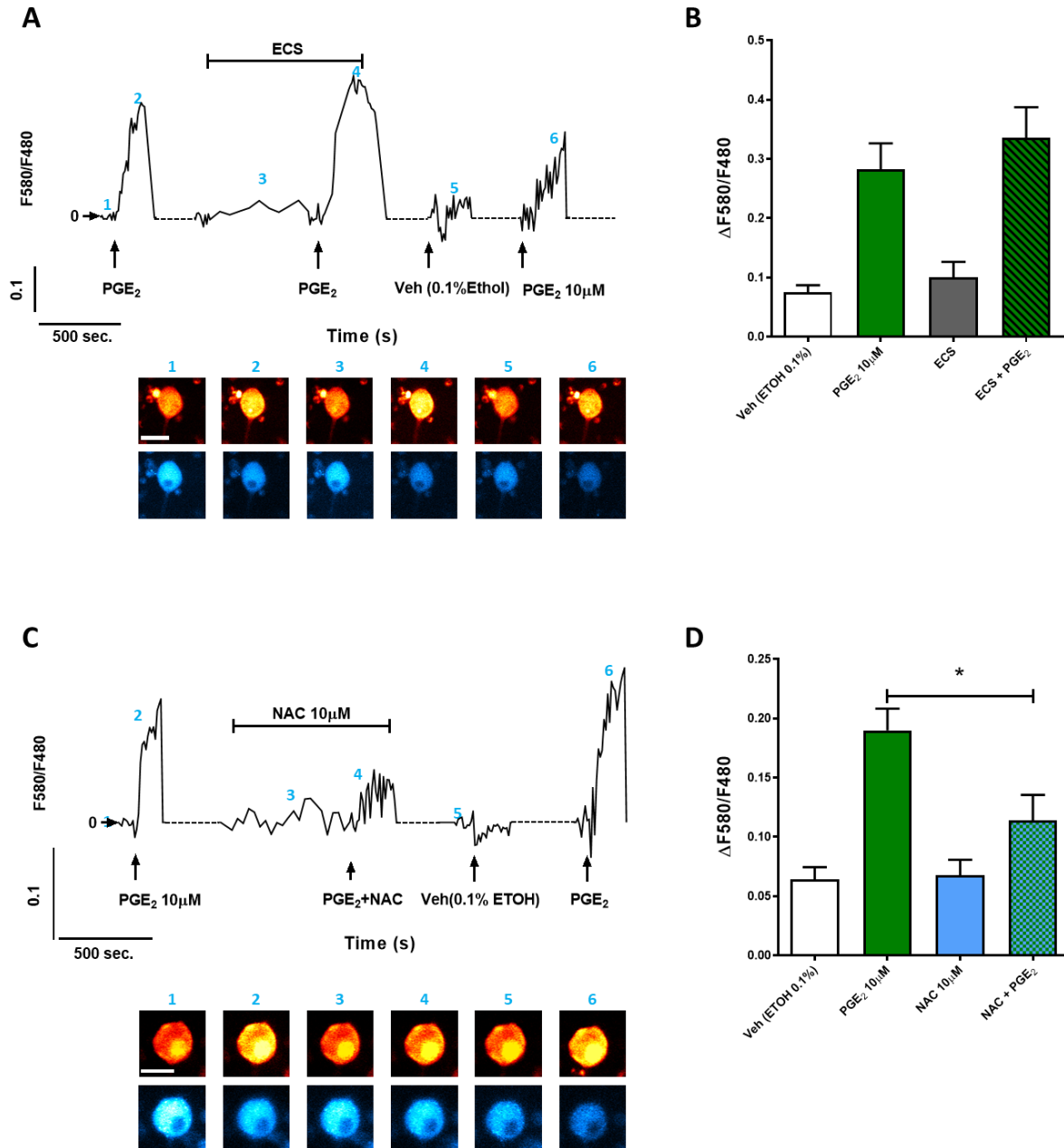


Figure 6.6 TRPA1 modality (PGE₂)-induced oxidative stress in guinea pig isolated airway specific neurons

A, C) Example traces of ROS formation induced by PGE₂ over time. When DHE is oxidized by ROS, it migrates from the cytosol where it emits blue light to the nucleus where the light sends upon binding to the DNA is red. The signal is shown as the pseudo ratio of the light emitted at 580nm (light coming from the nucleus zone) over the light emitted at 480 nm (light coming from the cytosol area). Drug injection and treatments are indicated underneath each trace respectively using arrows and time bars. Images were not recorded during the wash out periods (dashed lines) to avoid destruction of the dye by UV used to stimulate it to obtain the 480nm signal. Images of the light intensities recorded at 480nm and 580 nm with baseline corrected were extracted at the times indicated by the blue labels (1 to 6) to illustrate the oxidative signal (brighter nucleus, darker cytosol) induced prior to and after application of the agonists. The white bar on the images represent 50 μ m.

B, D) Summary bar graph showing the averaged oxidative stress responses induced by PGE₂. The response was determined as the peak ratio minus the baseline ratio and expressed as delta ratio ($\Delta F580/F480$). Data are

presented as mean $\pm$ S.E.M. * indicate $p < 0.05\%$ when tested with a paired t -test between test and control response. 21-28 primary airway neurons were harvested from 3-4 guinea pigs and used in this work.

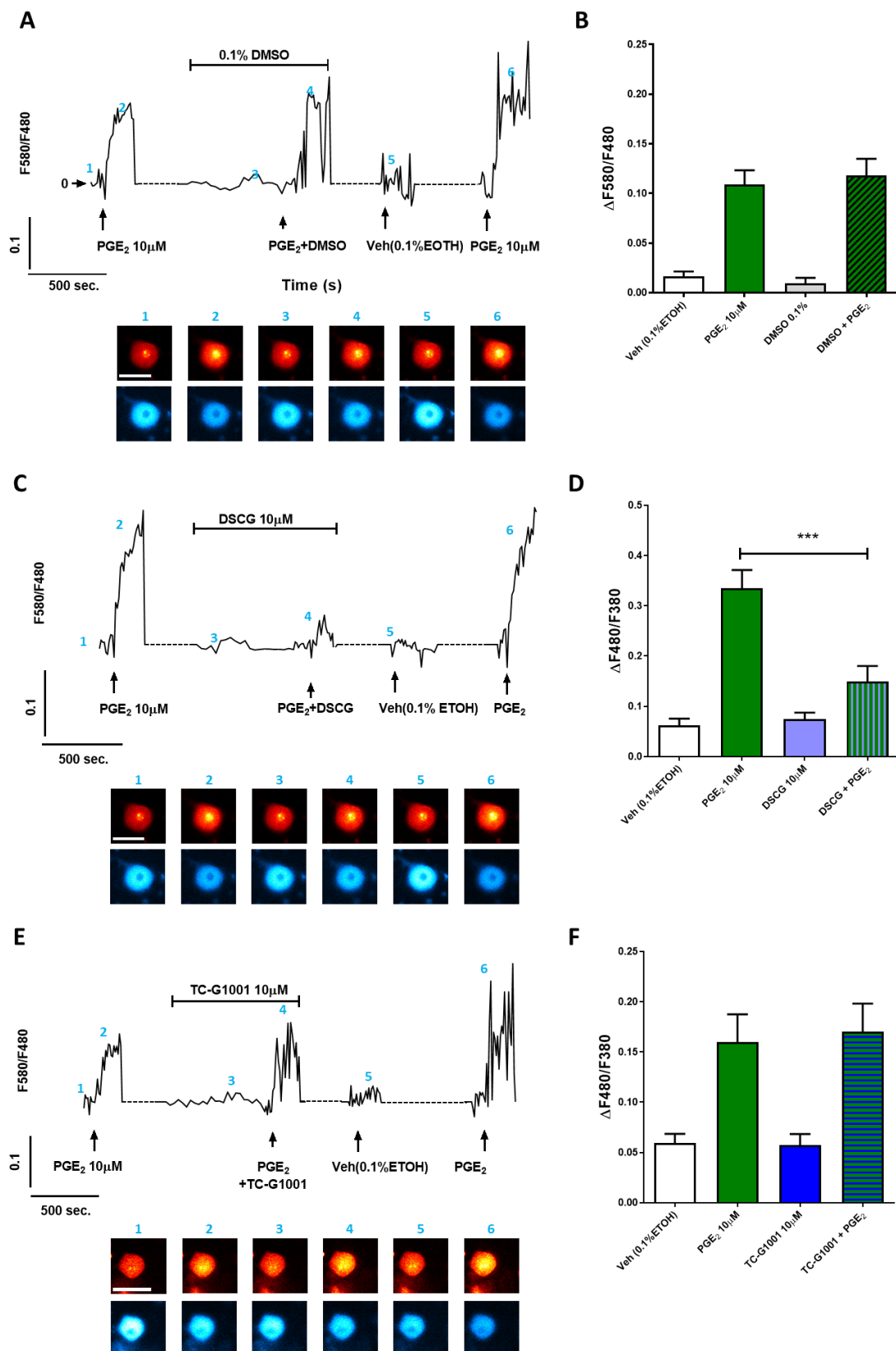


Figure 6.7 Effect of DSCG and GPR35 agonist on TRPA1 modality (PGE₂)-induced oxidative stress in guinea pig isolated airway specific neurons

A, C, E) Example traces of reactive oxygen species (ROS) formation in airway specific neurons over time. The signal is shown as the pseudo ratio of the light emitted at 580nm coming from the nucleus zone over the light emitted at 480 nm coming from the cytosol area. Drug injection and treatments are indicated underneath each trace respectively using arrows and time bars. Images of the light intensities recorded at 480nm and 580 nm with baseline corrected were extracted at the times indicated by the blue labels (1 to 6) to illustrate the oxidative signal (brighter nucleus, darker cytosol) recorded prior to and after application of the agonists. The white bar on the images represent 50 μ m.

B, D, F) Summary bar graph showing the averaged oxidative responses induced by PGE₂ alone and in presence of the compounds. The responses were determined as the peak ratio minus the baseline ratio and expressed as delta ratio ($\Delta F_{580}/F_{480}$). Data are presented as mean $\pm$ S.E.M. *** indicate $p < 0.005\%$ when tested with a paired t-test between test and control response. 34-39 primary airway neurons were harvested from 5 guinea pigs and used in this work.

6.3.5 Effect of antioxidants on TRPA1 mediated vagus nerve depolarisation

The data in the airway neurons would suggest that DSCG can reduce OS levels, which in turn might suggest this is how DSCG is attenuating TRPA1 responses that involve increases in intracellular oxidant stress, like PGE₂. Indeed, the respiratory Pharmacology group has previously shown that PGE₂ can cause the activation of sensory nerves through the activation of EP₃ receptors and the opening of both TRPA1 and TRPV1 channel¹³⁷. The TRPA1 component of the EP₃R driven response is assumed to involve an increase in intracellular OS. To strengthen this association, I wanted to use a modulator of a source of intercellular OS, NADPH oxidase. VAS2870 is a cell-permeable and specific NADPH oxidase (NOX) inhibitor that effectively suppresses ROS liberation²²⁹. The data showed that VAS2870 (5 μ M) was able to partially inhibit PGE₂-induced depolarisation (Figure 6.8 B). This level of inhibition was similar to that obtained with DSCG treatment. Further experiments performed in the presence of a TRPV1 blocker, showed that the PGE₂ response that was remaining was almost completely abolished. This indicates that the PGE₂ induced opening of TRPA1 is via the increase in NADPH oxidase induced intracellular OS. In a parallel experiment, I showed that VAS2870 did not modulate TRPV1 responses in this nerve preparation (data not shown).

Following this result, I then profiled the impact of the NADPH oxidase inhibitor against other stimuli that was modulated with DSCG, and not the GPR35 agonist. PGE₂ (10 μ M), hyperosmolar solution (+300 mOsm/L), bradykinin (3 μ M) induced depolarisation was inhibited by DSCG and VAS2780 to a similar level. This was also observed in rat tissue. This would suggest that DSCG can attenuate NADPHase linked activation of TRPA1 on sensory nerves.

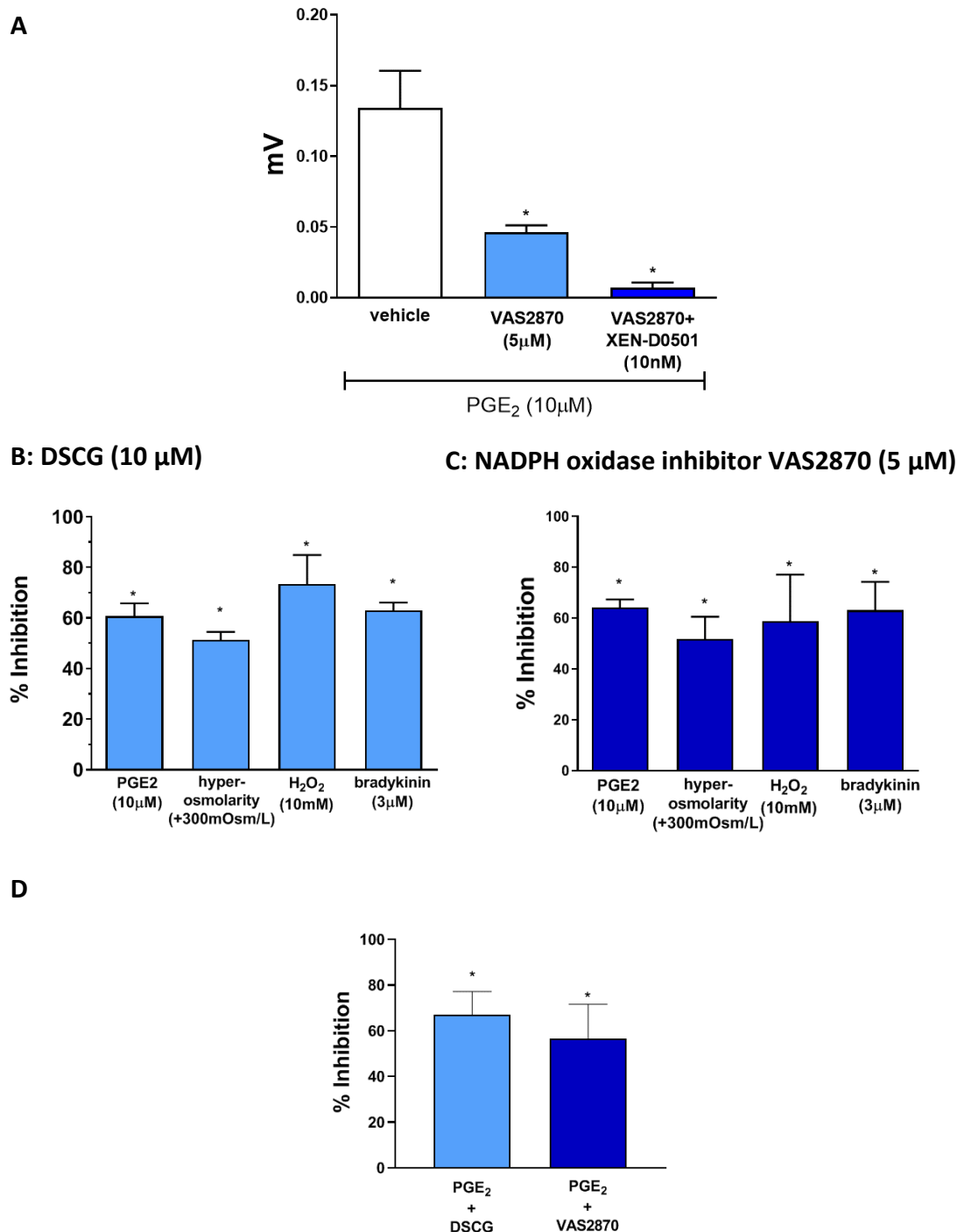


Figure 6.8 Effect of anti-oxidants on PGE₂-induced depolarisation of guinea pig and rat vagus nerve

A) Graphs showing effect of vehicle (0.01% v/v DMSO), VAS2870 (5 µM) or VAS2870 combining with XEN-D0501 (10 nM) on PGE₂ (10 µM) induced depolarisation in GP vagus nerve. Data displayed as mean ± S.E.M. depolarisation levels, n = 3-4. *p<0.05 comparing depolarisation levels before and after treatment using a paired t-test. B) Graphs showing effect of DSCG (10µM) on PGE₂ (10µM), hyperosmolar solution (+300 mOsm/L), bradykinin (3µM) and H₂O₂ (10mM) induced depolarisation in GP vagus nerve. C) Graphs showing effect of VAS2870 (5µM) on PGE₂ (10µM), hyperosmolar solution (+300 mOsm/L), bradykinin (3µM) and H₂O₂ (10mM) induced depolarisation in GP vagus nerve. D) Graph showing effect of DSCG (10µM) and VAS2870 (5µM) on PGE₂ (10µM) induced depolarisation in rat vagus nerve. Data displayed as mean ± S.E.M. percentage inhibition of

control responses, $n = 3-4$. $*p < 0.05$ comparing depolarisation levels before and after treatment using a paired t -test.

6.3.6 Effect of TRPV2 antagonists on TRPA1 mediated vagus nerve depolarisation

Having established that NADPH oxidase activity is involved in some TRPA1 driven responses, and that DSCG might have its effect via the blockade of this enzyme, I wanted to investigate the mechanism.

Activation of NADPH oxidase activity has been linked to the opening of TRPV2 channels²⁵⁵. A study has reported the involvement of TRPV2 in an osmotic stress-activated calcium influx; a response that involves NADPH oxidase²⁵⁵. Thus, the hypothesis is that DSCG could be modulating NADPH oxidase activity through an impact on TRPV2 and/or its signalling. To do this, three TRPV2 antagonists (tranilast, SKF96365 and NK-4) were employed^{215,220,252,253}.

I was able to mimic the effect of DSCG with three different TRPV2 antagonists (tranilast 10 μ M, SKF96365 10 μ M, NK-4 30 μ M). In Figure 6.9 A, all three TRPV2 antagonists have shown inhibitory effect on TRPA1 activator PGE₂-driven response on sensory nerve up to $54.5\% \pm 13.1\%$, $40.4\% \pm 7.1\%$, $54.9\% \pm 3.9\%$ respectively, similarly to DSCG. Whereas, as expected, the compounds did not impact on acrolein triggered responses (Figure 6.9 B). In a parallel experiment, I showed that both tranilast and SKF96365 inhibited TRPA1 activator H₂O₂-driven response on vagus nerve (data not shown).

Together these data suggest that DSCG can modulate intracellular activation of TRPA1 through the inhibition of NADPH oxidase activity via the modulation of TRPV2/signalling.

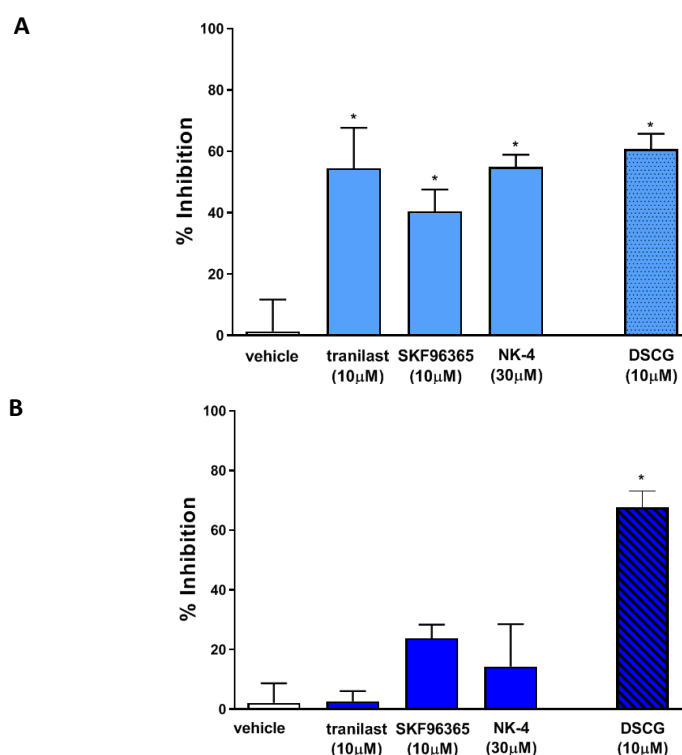


Figure 6.9 Effect of TRPV2 antagonists on PGE₂-driven depolarisation.

A) Graph showing effect of vehicle (0.1% v/v DMSO), tranilast (10 μ M), SKF96365 (10 μ M), NK-4 (10 μ M) and DSCG (10 μ M) on PGE₂ (10 μ M) induced depolarisation in GP vagus nerve. B) Graphs showing effect of vehicle (0.1% v/v DMSO), tranilast (10 μ M), SKF96365 (10 μ M), NK-4 (10 μ M) and DSCG (10 μ M) on acrolein (300 μ M) induced depolarisation in GP vagus nerve. Data displayed as mean $\pm$ S.E.M. percentage inhibition of control responses, $n = 3-4$. * $p < 0.05$ comparing depolarisation levels before and after treatment using a paired t-test.

6.4 Discussion

The initial aim of this chapter was to determine if DSCG or activation of GPR35 could modulate TRPA1 activity irrespective of how it is triggered. As discussed, TRPA1 ion channels can be opened by a range of endogenous and exogenous ligands. TRPA1 was known as an oxidative stress sensor and indeed most ROS (reactive oxygen species), RNS (reactive nitrogen species) and RCS (reactive carbonyl species) have been reported as TRPA1 activators^{152,19}. Components of diesel exhaust particles found in air pollution lead to mitochondrial ROS production, which activates TRPA1 on nociceptive C-fibers¹⁴⁵. PGE₂ and bradykinin activate airway sensory nerves via their EP₃R and B₂R respectively^{5,20}. Agonising these receptors leads to the opening of TRPA1 and TRPV1 ion channels^{5,20}. Around 50% of the activation of nerve fibres with PGE₂/BK is due to TRPA1, which in turn is due to intercellular production of oxidative stress. Hyper-osmotic solutions and cold temperatures have also been reported to trigger TRPA1, although the latter is unlikely to involve OS^{16, 21}. The temperature response is likely to be due to a conformational transition of the channel protein structure¹³².

Before I could investigate the impact of DSCG and GPR35 agonists, I wanted to demonstrate that, in my systems, the response was dependent on TRPA1. This was achieved using a selective TRPA1 antagonist. The data generated in rat, guinea pig and human vagal tissue showed that this range of stimuli could trigger robust responses and that TRPA1 plays a central role. Following this I profiled the impact of DSCG and a GPR35 agonist. DSCG inhibited sensory nerve activation induced by DEP-E, PGE₂, hyperosmolar solution, bradykinin and H₂O₂. Interestingly however, GPR35 agonist TC-G 1001 only inhibited DEP-E but not PGE₂, hyperosmolar solution, bradykinin and H₂O₂. Neither DSCG nor TC-G 1001 inhibited 10°C induced depolarisation. This last observation may be due to the mechanism by which cold temperature opens the ion channel.

Following the observation that there was a different inhibitory profile of DSCG and GPR35 on these alternate TRPA1 activators, the effect of the GPR35 antagonist CID2745687 was then evaluated to further confirm the role of this receptor. The antagonist was able to attenuate the inhibitory effect of DSCG and GPR35 agonist TC-G 1001 on acrolein and DEP driven responses but not PGE₂. This data helped confirm the divergence of effects of DSCG over GPR35 agonists. Figure 6.10 shows graphically the divergence and impacts of the two ligands.

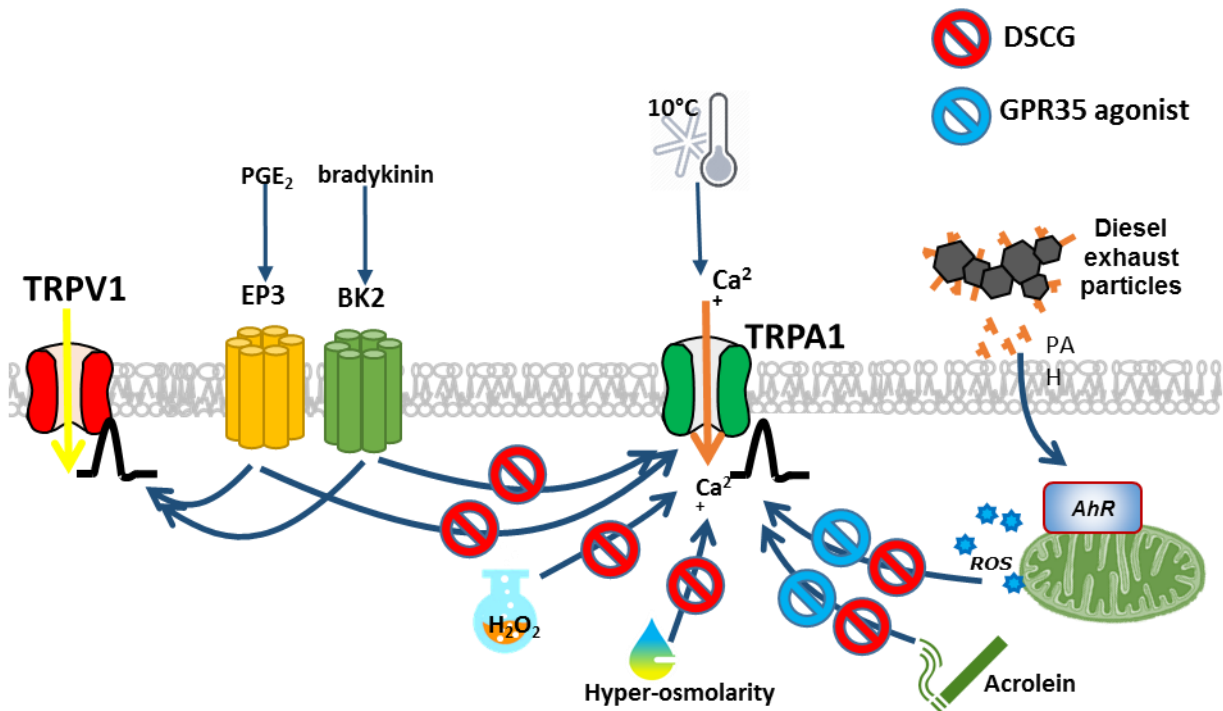


Figure 6.10 Schematic of the inhibition profile of DSCG and GPR35 antagonist TC-G 1001

Red stop sign indicates the TRPA1 activation that DSCG can inhibit; Blue stop sign indicates the TRPA1 activation that GPR35 antagonist TC-G 1001 can inhibit.

To further investigate these observations I used primary airway sensory neurons. Using these cells, I confirm that DSCG but not TC-G 1001 inhibits PGE_2 -mediated responses. Further, I showed that PGE_2 stimulation did trigger an increase in intracellular ROS. And this ROS signal was attenuated with DSCG but not the GPR35 agonist. As DSCG has been reported to possess some anti-oxidant properties, this data led me to the hypothesis that DSCG is somehow capable of modulating the PGE_2 induced intracellular ROS¹⁹⁸. To investigate this hypothesis, I decided to explore firstly the mechanism by which EP_3 R activation increases intracellular OS and ways in which DSCG could be impacting on this.

The data showed that a known mechanism for increasing intracellular ROS, NADPH oxidase was involved in PGE_2 triggered opening of TRPA1 ion channels. The data showed that VAS2870 was able to inhibit PGE_2 -induced depolarisation. This level of inhibition was similar to that obtained with DSCG treatment. Following this result, I then profiled the impact of the NADPH oxidase inhibitor against other stimuli that was modulated with DSCG, and not the GPR35 agonist. PGE_2 , hyperosmolar solution, bradykinin induced depolarisation were all inhibited by DSCG and VAS2780 to a similar level. This would suggest that DSCG can attenuate NADPHase linked activation of TRPA1 on sensory nerves.

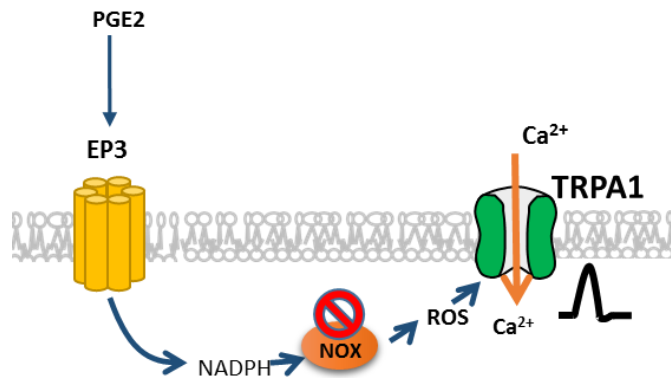


Figure 6.11 Schematic of the inhibition of NADPHase linked activation of TRPA1 on sensory nerves (PGE₂ as an example)

Having established a role for NADPH oxidase in the DSCG sensitive responses, my next step was to investigate the mechanism. Activation of NADPH oxidase has been linked to opening of the TRPV2 channel²⁵⁵. TRPV2 was firstly identified in the lab of David Julius as a close homolog of TRPV1²⁵⁶. TRPV2 is expressed in the lymph nodes, spleen, lung, appendix, placenta and highly in human lungs²⁵⁷. It has been reported to be on sensory neurons, as well as being distributed in the brain and spinal cord²⁵⁸. Furthermore, a study has suggested that TRPV2 is linked to childhood asthma; the expression of TRPV2 gene was significantly increased in asthmatic children compared with healthy subjects²⁵⁹. Another study has reported the involvement of TRPV2 in an osmotic stress-activated calcium influx pathway in dystrophic cardiomyopathy, and NADPH oxidase activities were involved in this process. To test the role of TRPV2 in my system, I employed three structurally different antagonists: tranilast, SKF96365 and NK-4^{215,220,252,253}. All three TRPV2 antagonists inhibited PGE₂-driven responses; mimicking the effects of DSCG and VAS2780. This suggests that DSCG may attenuate NADPH oxidase activities through the inhibition of the TRPV2 channel, either directly or through the modulation of the signalling pathway. Furthermore, the TRPV2 antagonists did not modulate GPR35 agonist-sensitive responses like acrolein. This adds further weight to the idea that DSCG may be acting via different mechanism to suppress TRPA1 (Figure 6.12). Together this data may suggest that DSCG can modulate intracellular activation of TRPA1 through the blockade of TRPV2 channel/signalling and subsequent reduction of NADPH oxidase activity.

I therefore reject my hypothesis 'DSCG and GPR35 inhibit TRPA1 activation on airway sensory nerves by a broad range of TRPA1 modalities' and suggest instead that DSCG can inhibit activation driven by indirect TRPA1 activators through the inhibition of oxidative stress. Together this would suggest that DSCG can elicit inhibitory effects on airway sensory nerves via two distinct mechanisms: direct activation of the channel can be inhibited via a GPR35 dependent mechanism, and indirect NADPH-oxidase driven activation can be inhibited utilising the antioxidative effect of DSCG and inhibition of

TRPV2. This impact of DSCG could be responsible for some of its beneficial effects observed in the clinic and may highlight novel targets for drug discovery.

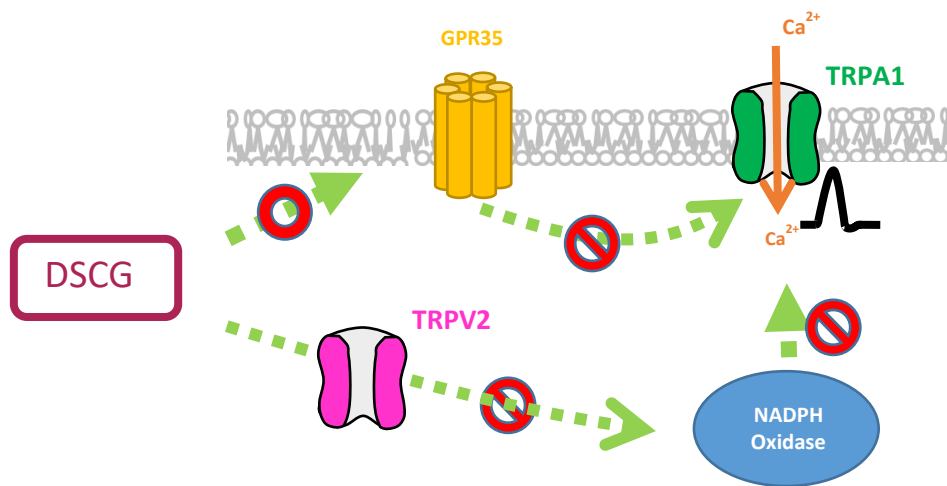


Figure 6.12 Schematic of the mechanism of DSCG on TRPA1-driven sensory nerve activities

Chapter 7 Summary and Future Studies

7.1 Summary

Asthma is a disease with increasing global prevalence and patients typically suffer from symptoms such as wheezing, breathlessness, chest tightness and cough. Although asthma can be controlled under current treatments, adherence to them is poor and inhaler use often insufficient³⁸. Around 60% of asthma patients are uncontrolled across the GINA steps, which has a big impact on their health-related quality of life³⁹. Therefore, these patients have an urgent need for new therapeutics.

Disodium Cromoglycate (DSCG) has been utilised as an asthma therapy for a number of years as it is known to modulate symptoms with a favourable safety profile¹⁶¹. However, its use is limited because of its short duration of action seen evidenced by its pharmacokinetic profile. The mechanism of action of DSCG was thought to be through stabilising mast cells but other mast cell stabilising compounds have failed as asthma therapeutics¹⁸⁵, indicating that the beneficial effects of DSCG may be via alternative mechanisms. If one could determine the mechanism of action of DSCG, it may be possible to develop a novel therapy with a longer PK profile and thus be a more useful treatment. Therefore, the aim of this thesis is to investigate the mechanism of action of DSCG.

Allergen challenge in asthmatic patients results in bronchoconstriction which occurs in two stages; the Early Asthmatic Response (EAR) which is immediately after challenge²³⁰ and the Late Asthmatic Response (LAR) occurring approximately 3-8 hours following allergen exposure²⁷. The LAR is a characteristic key feature of asthma and is often used in clinical research as an end point to study new therapeutic options. Within the Respiratory Pharmacology group, the LAR has been modelled successfully in Brown Norway rats, with which they have shown that the LAR is an airway sensory nerve mediated parasympathetic reflex^{27,231}. Previous studies in the group have also shown that in this rat model, allergen challenge triggers airway sensory nerves via the activation of TRPA1 channels²⁷. To confirm these results, in my thesis a second TRPA1 antagonist, Janssen 130, was investigated in this model where Janssen 130 significantly inhibit the LAR response in the BN rat, providing further confirmation to the key role of TRPA1 ion channel in the LAR. Furthermore, following this confirmation, DSCG was also shown to have an inhibitory effect on the LAR, indicating that it may have an effect on the TRPA1 ion channel.

Previous data suggested that DSCG was able to inhibit TRPA1 induced activation of nerves from the GI tract²³⁵. Having shown that TRPA1 is required for the LAR response, and that DSCG can inhibit the LAR, this led me to postulate that DSCG may attenuate asthma symptoms by modulating TRPA1 on airway sensory nerves. To test this hypothesis, a range of systems were used, including *in vitro* model of vagal sensory nerve activation, *in vitro* model of airway specific neuron activation, *in vivo* sensory nerve recordings and *in vivo* cough model. Initially I employed the isolated vagus nerve preparation because

it is a more amenable technique to begin my investigations and that human tissue can be used to translate my findings. DSCG inhibited TRPA1 agonist acrolein induced depolarisation in rat, guinea pig and in human isolated vagus nerves. This suggests a novel mechanism of action for DSCG beyond its known function as a mast cell stabiliser, and also further implicates TRPA1 as a key driver in asthma symptoms such as the LAR. Whilst the inhibitory effect of DSCG on TRPA1-mediated nerve activation was demonstrated in isolated whole vagus nerve segments, it is known that the vagus nerve innervates not only the airways, but also other visceral organs²⁴⁰. Therefore, in order to clarify that DSCG exerts its inhibitory effect on TRPA1 on airway sensory nerves, vagal ganglia neurons specifically innervating the airway were isolated and intracellular calcium levels assessed as a marker of neuron activation. Acrolein was able to induce increases in intracellular Ca^{2+} in airway specific neurons, and this activation by acrolein was inhibited by DSCG in both guinea pig and rat airway neurons. These results demonstrated that DSCG can inhibit TRPA1 initiated airway nerves.

By using *in vitro* techniques to evaluate DSCG, I confirmed its inhibitory effects on TRPA1-driven airway nerve activities. However, these techniques were only investigating *in vitro* functions, and neither assesses the effect of DSCG on a nerve terminal nor actual nerve firing. The expression of receptors and signal transduction pathways on the nerve trunk and/or ganglia cell bodies may not be the same as on the nerve endings. Therefore, to further investigate this I employed two *in vivo* systems: afferent airway sensory nerve recording from anaesthetised animals and induced cough in the conscious guinea pig. Acrolein aerosolised into the lungs of anaesthetised animals triggers firing of C-fibres. This firing was significantly inhibited following administration of DSCG. This data confirms that DSCG, when applied at nerve terminals, is capable of inhibiting action potential firing induced by TRPA1 in GP C-fibres *in vivo*. Similarly, aerosolised acrolein triggers coughing in conscious guinea pigs. The data showed that pre-treatment with the selective TRPA1 antagonist, Janssen 130, or DSCG significantly inhibited the cough response. The anti-tussive impact of DSCG has been reported by others^{179,189,241}. The results in this chapter has suggested that DSCG attenuates the LAR via inhibition of TRPA1 ion channels on airway sensory nerves.

The next aim was to determine how DSCG could inhibit this ion channel. A previous study has reported that DSCG is a potent agonist of the G protein-coupled receptor 35 (GPR35)^{191,192}. Furthermore, GPR35 agonists have been suggested to regulate calcium signaling in neurons¹⁹⁴. Therefore, my next step was to investigate the role of GPR35 in DSCG-induced inhibition of TRPA1 sensory nerve activation. By using selective GPR35 agonists and antagonists, I hope to determine if GPR35 activation could replicate the effect of DSCG, and also whether a GPR35 antagonist could block the impact of DSCG. A range of systems, including *in vitro* model of vagal sensory nerve activation, *in vitro* model of airway

specific neuron activation, *in vivo* sensory nerve recordings and *in vivo* LAR model were used. Initially in the isolated vagus nerve preparation, a GPR35 agonist was shown to inhibit the TRPA1 agonist acrolein driven response, and a GPR35 antagonist blocked the inhibitory effect of DSCG on TRPA1 responses. I then used calcium imaging of airway-specific neurons *in vitro* to confirm that the observed functional effects were present in airway innervating neurons. Following this, in an *in vivo* system, a GPR35 agonist was shown to inhibit single fibre action potential firing induced by TRPA1; and a GPR35 antagonist was shown to block the inhibitory effect of DSCG on single fibre firing induced by TRPA1. In the model of the LAR, a GPR35 antagonist reversed the inhibitory effect of DSCG, in addition to a GPR35 agonist inhibiting the LAR. Therefore, there is strong evidence to suggest that DSCG attenuates the LAR via acting on GPR35.

Interestingly, an anti-inflammatory effect was also observed with the GPR35 agonist HTL 227477. HTL 227477 reduced the number of eosinophils, neutrophils, lymphocytes and macrophages in the BAL fluid. A similar anti-inflammatory effect was seen with DSCG. This would suggest that GPR35 activation could have additional beneficial anti-inflammatory effects, which can be investigated in the future.

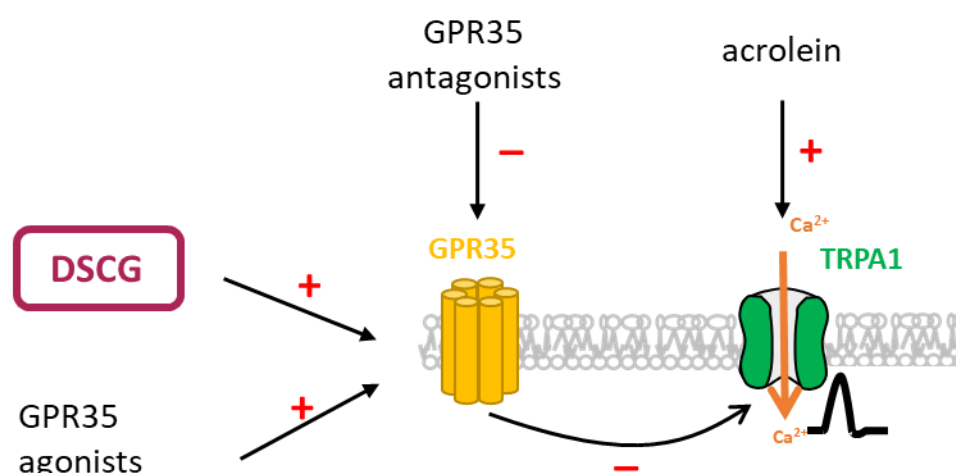


Figure 7.1 Schematic of GPR35 mechanism on TRPA1-induced airway sensory nerve activation

So far, all the investigations have been carried out utilising the TRPA1 agonist acrolein. As reported, TRPA1 is an ion channel which can be activated by a series of stimuli, such as prostaglandin E₂ (PGE₂), bradykinin (BK), reactive oxygen species, and increases in osmolarity, along with cold temperatures¹⁴⁸. In my next step I wanted to assess if activation of GPR35 could modulate a range of TRPA1 stimuli.

As discussed, TRPA1 ion channels can be opened by a range of endogenous and exogenous ligands. Therefore PGE₂, BK, H₂O₂, diesel exhaust particles (DEP-E), hyperosmolar solution (+300 mOsm/L) and 10°C were used in this chapter. Before I could investigate the impact of DSCG and GPR35 agonists, a selective TRPA1 antagonist was used to demonstrate the response was dependent on TRPA1 in the

system. Following this, the impact of DSCG and GPR35 agonist TC-G 1001 was profiled. DSCG inhibited sensory nerve activation induced by DEP-E, PGE₂, hyperosmolar solution, BK and H₂O₂. Interestingly however, GPR35 agonist TC-G 1001 only inhibited DEP-E but not PGE₂, hyperosmolar solution, BK and H₂O₂. Following this observation, the effect of the GPR35 antagonist CID2745687 was then evaluated to further confirm the role of this receptor. The antagonist was able to attenuate the inhibitory effect of DSCG and GPR35 agonist TC-G 1001 on acrolein and DEP-E driven responses but not PGE₂. All the data together confirm the divergence of effects of DSCG over GPR35 agonists.

Primary airway sensory neurons were used to further investigate observations. It was confirmed that DSCG but not TC-G 1001 inhibits PGE₂-mediated responses. Further, it was shown that PGE₂ stimulation did trigger an increase in intracellular ROS. Interestingly this ROS signal was attenuated with DSCG but not the GPR35 agonist. As DSCG has been reported to possess some anti-oxidant properties, this data led me to the hypothesis that DSCG is somehow capable of modulating the PGE₂ induced intracellular ROS¹⁹⁸. To investigate this hypothesis further, I decided to firstly explore the mechanism by which EP₃R activation increases intracellular OS and if DSCG could be impacting on this.

The data showed that a known mechanism for increasing intracellular ROS, NADPH oxidase was involved in PGE₂-triggered opening of TRPA1 ion channels. NADPH oxidase inhibitor VAS2870 was able to inhibit PGE₂-induced depolarisation in a similar inhibitory level of DSCG treatment. Following this, I then profiled the impact of the NADPH oxidase inhibitor against other stimuli that was modulated with DSCG, and not the GPR35 agonist. PGE₂, hyperosmolar solution, BK induced depolarisation were all inhibited by DSCG and VAS2870 to a similar level. This would suggest that DSCG can attenuate NADPHase linked activation of TRPA1 on sensory nerves.

Having established a role for NADPH oxidase in the DSCG sensitive responses, my next step was to investigate the mechanism. Activation of NADPH oxidase has been linked to opening of the TRPV2 channel²⁵⁵. To test the role of TRPV2, three structurally different antagonists: tranilast, SKF96365 and NK-4^{215,220,252,253} were used where all three TRPV2 antagonists inhibited PGE₂-driven responses; mimicking the effects of DSCG and VAS2870. This suggests that DSCG may attenuate NADPH oxidase activities through the inhibition of the TRPV2 channel, either directly or through the modulation of the signalling pathway. Together this data may suggest that DSCG can modulate activation of TRPA1 through the blockade of TRPV2 channel/signalling and subsequent reduction of NADPH oxidase activity.

In 2017, a novel formulation of DSCG was shown to reduce coughing in patients with IPF⁵⁰, and the anti-tussive mechanism of DSCG was not studied. The mechanism in my thesis may help to explain its efficacy. Oxidative stress is widely recognized as a central feature of IPF. Antioxidant therapy has been

proposed as an effective treatment for IPF²⁶⁰. Therefore, DSCG might reduce cough in IPF patients via antioxidative effect and subsequently modulating airway sensory nerve function.

In summary, I have demonstrated that DSCG can elicit inhibitory effects on airway sensory nerves via two distinct mechanisms: direct activation of the channel can be inhibited via a GPR35 dependent mechanism, and indirect NADPH-oxidase driven activation can be inhibited utilising the antioxidative effect of DSCG and inhibition of TRPV2. The work within my thesis has therefore uncovered the important role of GPR35, TRPV2 and NADPH oxidase activity in the mechanism of action of DSCG by using pre-clinical models. This impact of DSCG could be responsible for some of its beneficial effects observed in the clinic and may highlight novel targets for drug discovery.

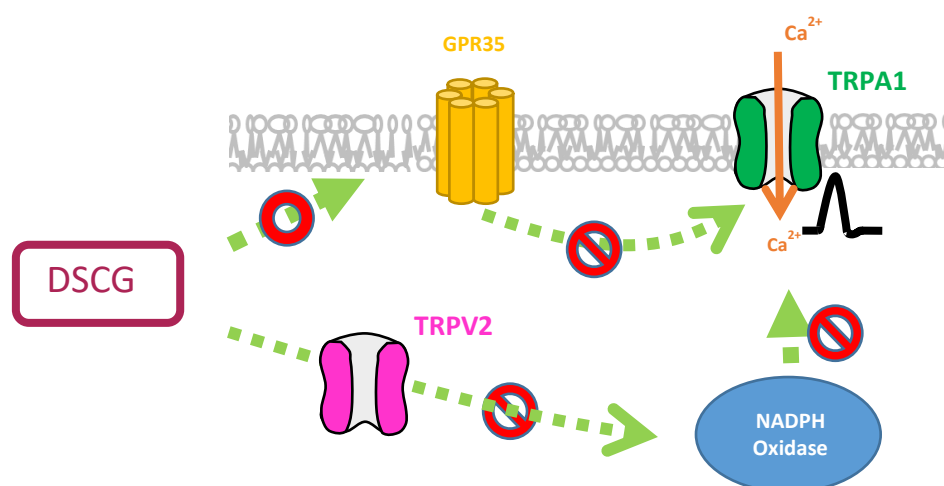


Figure 7.2 Schematic of the mechanism of DSCG on TRPA1-driven sensory nerve activities

7.2 Limitation of Thesis

Within this thesis there are several limitations associated with the experimental techniques/tools employed, some of which have been described previously.

It is of note that the use of OVA driven models of allergic asthma has received criticism for several reasons. Firstly OVA is rarely an antigen for humans to develop asthma as such it is not clinically relevant. Secondly the route of administration for antigen sensitisation is not mimicking the topical routes which is usually how humans develop sensitisation clinically¹¹⁰. Models utilising more disease relevant allergens such as HDM are currently under development. However, unlike the OVA model, HDM model has not been fully studied and the role of immune cell populations and inflammatory mediators still needs to be investigated. In addition, it has been shown that a single topical dose of

HDM in the rat was able to induce asthma-like airway inflammation, suggesting that initial inflammation in this model is innate in nature¹¹⁷. Moreover, none of these recently developed HDM-driven models display the LAR^{261–263}. As such, HDM driven model was not used in this thesis.

The use of the isolated vagus nerve recording technique allowed for a cost effective, relatively high throughout pharmacological evaluation of sensory nerve activity. In addition, results were confirmed in donor human vagal tissue indicating translation to the clinic. However, as depolarisation is measured from the nerve trunk, this is not a true measure of what may occur *in vivo* as receptors and ion channels may be in a different conformation as found at the nerve terminals. Furthermore, the vagus nerve can innervate other organ systems including the heart and the GI tract. This means that assessment of the activity of specifically airway terminating nerves is not possible in this technique.

This problem was circumvented via the use of the *in vivo* single fibre afferent recording model in guinea pig and rat where responses involve interactions at the nerve terminals and therefore does allow for action potential assessment. However, this technique is relatively labour intensive and only one nerve fibre can be examined in a single animal. Therefore, the effect of TRPV2 and anti-oxidant was not tested in this system but would be desirable in future work.

In this thesis, rat and guinea pig were the animal species for investigation. Mouse, as a common and widely used species, was not used. This is due to the fact that in the early stage of the investigation, DSCG did not show any inhibitory effect on TRPA1-induced responses in mouse vagus nerve. Consequently, it is not possible to use GPR35 or TRPA1 knock-out (KO) mouse during the investigation, which would have provided a platform to determine the role of these receptors in addition to pharmacological intervention.

As discussed, TRPA1 ion channels can be opened by a range of endogenous and exogenous ligands. TRPA1 was known as an oxidative stress sensor and indeed most ROS (reactive oxygen species), RNS (reactive nitrogen species) and RCS (reactive carbonyl species) have been reported as TRPA1 activators^{152,19}. In this thesis, only ROS production was investigated. To profile a full picture of DSCG's antioxidant effect, RNS (reactive nitrogen species) and RCS (reactive carbonyl species) still need to be investigated.

7.3 Future work

7.3.1 Future investigation of the GPR35-dependent mechanism

7.3.1.1 Explore the downstream signalling pathways associated with GPR35

There is little known about the mechanism by which the activation of GPR35 can modulate TRPA1 driven responses in airway sensory innervation. It has been previously reported that activation of GPR35 triggers a Gi dependent inhibition of N-type calcium channels in rat neurons¹⁹⁴. Other studies have reported that Gi can be inactivated by dosing rats with pertussis toxin²⁶⁴. Therefore, in future experiments, Brown Norway rats could be dosed with pertussis toxin and their vagus nerves harvested to test whether the inhibitory effect of DSCG on TRPA1 induced nerve depolarisation was affected.

7.3.1.2 Explore the effect of GPR35 on inflammation

In this thesis an anti-inflammatory effect was observed with the GPR35 agonist HTL 227477. HTL 227477 reduced the number of eosinophils, neutrophils, lymphocytes and macrophages in the BAL fluid. Therefore, it could be interesting to study the role of GPR35 on these inflammatory cells. To start with, gene and protein expression levels of TRPA1 and GPR35 would be assessed. If these receptors were shown to be present, then the functional impact of GPR35 could be investigated using *in vivo* asthma models and cell based assays.

7.3.1.3 Explore the role of GPR35 in cough

Unfortunately, due to time constraints, I was unable to assess whether HTL 227477 was also capable of inhibiting the cough reflex in conscious guinea pigs. I would initially investigate whether HTL 227477 was capable of inhibiting TRPA1 mediated C-fibre firing in the guinea pig *in vivo*, following which I would carry out experiments utilising HTL 227477 at various doses against acrolein induced cough in the guinea pig. This would confirm the role of DSCG and GPR35 in airway reflexes, and is also important as DSCG has been shown to reduce cough in IPF patients²⁶⁰.

7.3.2 Future investigation of the anti-oxidative stress mechanism

7.3.2.1 Explore the effect of DSCG on the oxidative stress triggered by other stimuli

Other oxidative stress stimuli should be investigated to see if they are all susceptible to inhibition and conform to the anti-oxidative stress mechanism proposed to be partially responsible for the inhibitory effect of DSCG. In this thesis, only the TRPA1 activator PGE₂ was investigated but not BK, H₂O₂, DEP-E, hyperosmolar solution. To support this conclusion, it would be desirable to use other stimuli in the oxidative stress imaging system to test their ability to trigger oxidative stress, and then investigate the effect of DSCG.

As discussed, the TRPA1 ion channel can be opened by a range of endogenous and exogenous ligands. TRPA1 is known as an oxidative stress sensor and indeed most ROS (reactive oxygen species), RNS (reactive nitrogen species) and RCS (reactive carbonyl species) have been reported as TRPA1 activators^{152,19}. In this thesis, only ROS production was investigated. To gain a full picture of DSCG's antioxidant effect, RNS (reactive nitrogen species) and RCS (reactive carbonyl species) need to be investigated using similar protocols.

7.3.2.2 Explore the impact of TRPV2

Experiments are also needed to further investigate the role of the TRPV2 in the mechanism of action of DSCG as this channel is poorly studied. Ideally a FLIPr assay should be used to investigate the possible interaction between DSCG and TRPV2 channel. Following that oxidative stress imaging systems should be employed to study the effect of TRPV2 antagonists after confirming that all the TRPA1 agonists can trigger oxidative stress (described in 7.3.2.1). Afterwards, *in vivo* studies could be employed to study the effect of a TRPV2 antagonist on TRPA1-driven single fibre action potential firing to determine the impact of TRPV2 on airway sensory nerve activation. Eventually, the rat LAR model could be used to test if TRPV2 can modulate the LAR.

The signalling between TRPV2 and NADPHase would also shed more light on the mechanism of action. . NADPH oxidase is a membrane-bound enzyme complex that faces the extracellular space. It has a series of isoforms such as NOX1, NOX2 etc²⁶⁵. There are limited data and reports on the interaction between the NOX isoforms and TRPV2 channel in the literature and so experiments utilising specific NOX inhibitors would be useful in dissecting the mechanism.

7.3.3 Concluding Remarks

In this thesis I have demonstrated that DSCG can inhibit TRPA1-mediated activation of airway sensory nerves, in rat guinea pig and human tissue. This inhibitory effect was shown to be via two main mechanisms dependent on stimulus: the activation of the orphan GPCR GPR35, and also through an antioxidant mechanism involving inhibition of the TRPV2 ion channel. I have therefore completed one of the aims of my thesis by determining the mechanism of action and potentially uncovering novel mechanisms to target for future asthma therapeutics.

A further aim of this thesis was to profile an analogue of DSCG with an improved pharmacokinetic profile. I demonstrated that HTL 227477, a selective GPR35 ligand has an inhibitory effect on the TRPA1-mediated sensory nerve activation, the LAR and the inflammation. This compound appeared both potent and selective, and although further work is required to determine its full PK profile to determine the possible dosing regimen, HTL 227477 could be a potential candidate as an analogue of DSCG to be used as an asthma therapeutic.

This thesis has demonstrated a brand-new alternative theory to explain the mechanism of action of DSCG as an asthma therapy which is not linked to mast cell stabilisation. This work may also help to explain the beneficial effect of DSCG in cough in patients with idiopathic fibrosis⁵⁰.

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Appendix

The table below outlines the chemicals used throughout this thesis.

Drug/Reagent	Source
Acrolein	Sigma-Aldrich (UK)
Albumin from Chicken Egg White (OVA)	Sigma-Aldrich (UK)
Alum TM	ThermoFisher Scientific (US)
Budesonide	Sigma-Aldrich (UK)
Capsaicin	Sigma-Aldrich (UK)
Disodium Cromoglycate	Sigma-Aldrich (UK)
DMSO	Sigma-Aldrich (UK)
Dnase	Roche Diagnostics (Germany)
ESC solution (mM: NaCl 136, KCl 5.4, MgCl ₂ 1, NaH ₂ PO ₄ 0.33, HEPES 10, D-Glucose 10, CaCl ₂ 2.5; pH adjusted to 7.4 using NaOH 1M stock solution)	Sigma-Aldrich (UK)
Eosin-Phloxine solution	Sigma-Aldrich (UK)
Ethanol	VWR (UK)
FBS	Invitrogen (UK)
Formaldehyde	Sigma-Aldrich (UK)
Glycopyrrolate	Sigma-Aldrich (UK)
HBSS (phenol red: 10.62 mg/L; NaHCO ₃ : 350 mg/L; Hanks' salts (2% CO ₂))	Sigma-Aldrich (UK)
Isoflurane	Abbott laboratories (UK)
Janssen130	Almirall (Spain)
Krebs-Henseleit (KH) solution (mM: NaCl 118; KCl 5.9; MgSO ₄ 1.2; CaCl ₂ 2.5; NaH ₂ PO ₄ 1.2; NaHCO ₃ 25.5 and glucose 5.6; pH 7.4)	Sigma-Aldrich (UK)
Kynurenic Acid	Sigma-Aldrich (UK)
Medical Oxygen	BOC Industrial Gases (UK)
methycellulose	Sigma-Aldrich (UK)
ML145 (GPR35 inhibitor)	Sun Pharma Ltd (India)
Pamoic Acid	Sigma-Aldrich (UK)
PBS	Sigma-Aldrich (UK)
Saline	Fresenius Kabi (UK)
Sodium pentobarbitone	Merial Animal Health (UK)
Tiotropium Bromide	Sigma-Aldrich (UK)
Tween80	Sigma-Aldrich (UK)
Urethane	Sigma-Aldrich (UK)
Vecuronium Bromide	Sigma-Aldrich (UK)
XEND0501	Ario Pharma Ltd (UK)
Zaprinast	Sigma-Aldrich (UK)