

AN INVESTIGATION INTO THE ROLE OF THE ION CHANNEL TRPV4 IN THE ACTIVATION OF AIRWAY SENSORY NERVES AND THE COUGH REFLEX

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2015

**A thesis submitted for the Degree of Doctor of Philosophy
in the Faculty of Medicine, Imperial College London**

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Thesis Abstract

The TRPV4 channel is a member of the Transient Receptor Potential family of ion channels and is known to be expressed on airway smooth muscle, macrophages and epithelial cells within the lung. Whilst it has been reported that TRPV4 is present on sensory nerves, its role in modulating the function of airway sensory nerves and the cough reflex remains unexplored. The aim of this thesis was therefore to determine a role for TRPV4 in sensory nerve activity and the cough reflex.

Using an *in vitro* model of vagal sensory nerve activation and an *in vivo* guinea pig cough model, it was established that activation of TRPV4 by selective ligands activates airway sensory nerves and cause cough. Calcium imaging of isolated neurons and airway single fibre recordings determined that this was through activation of a different population of nerve fibres than those activated by TRPV1 and TRPA1 ligands, which are both known to activate sensory nerves and cause cough.

Calcium flux and fibre firing demonstrated a delay prior to activation by a TRPV4 agonist, suggesting an indirect mechanism of action. Activation of TRPV4 has been linked to ATP release in several cell types, and therefore it was hypothesised that activation of TRPV4 on the nerves leads to ATP release and subsequently activation of airway sensory nerves. The purine receptor P2X3, which is activated by ATP, is expressed on airway sensory nerves, and a selective P2X1/3 agonist caused depolarisation of the vagus nerve and firing of Aδ single fibres *in vivo*. Further, TRPV4 induced activation of sensory nerves *in vitro* and cough *in vivo* was inhibited following the administration of a selective P2X3 antagonist.

This data would suggest that the activation of airway sensory nerves by TRPV4 ligands causes ATP release which then activates P2X3 possibly present on the same subset of sensory nerves, to cause cough. The work within my thesis has therefore uncovered TRPV4 as a modulator of airway sensory nerves and associated functions such as cough, which exerts its actions via a novel mechanism of action and as such is a good candidate for possible anti-tussive therapies.

Acknowledgements

First and foremost I would like to thank my supervisors Dr Mark Birrell and Professor Maria Belvisi for their support, guidance, encouragement and scientific expertise offered throughout my PhD. I am extremely grateful to them both for making my PhD a challenging yet rewarding and thoroughly enjoyable experience. I would also like to thank the NHLI for funding my studentship.

My thanks also go to the Respiratory Pharmacology group past and present for their help, support, advice and friendship over the past 4 years, and of whom all have helped make my PhD a great experience. In no particular order I would like to thank Dr. Sarah Maher, Dr. Mike Wortley, Dr. Eric Dubuis, Katie Baker, Dr. Victoria Jones, Dr. Liang Yew-Booth, Dr. James Buckley, Dr. Megan Grace, Ryan Robinson, Dr Matt Baxter, Dr Nicole Dale, Bilel Dekkak and Abdel Dekkak. It has been a real pleasure working with all of you, and thank you all for making the PhD process so enjoyable. Special thanks also to Dr. John Adcock for his many hours carrying out single fibre experiments, and also for his careful proofreading of this thesis. I would also like to thank Dr Anthony Ford from Afferent Pharmaceuticals for the provision of the compound AF-353 used within this thesis.

I would also like to thank my friends and family for their continued support and encouragement throughout the PhD process. To my gym buddies (Sarah, Victoria and Liang), and also to my PhD buddy Katie I would like to particularly thank for your friendship and for providing much needed stress-relief at times. Big thanks also to Frankie, Lorien, Amelia, Hayley and Bethia for your continuous encouragement and friendship throughout my PhD. To my family; Mum, Dad, Nicola, Josh and Jon thank you for your unconditional support, love and encouragement throughout this process. Last but certainly not least my love and thanks go to Nick for his unending patience, love and understanding throughout my PhD, and for never failing to make me smile.

Statement of Originality

The work contained in this thesis is my own work, except where otherwise indicated and referenced appropriately.

The concentration response to GSK1016790a in the nodose ganglia (Chapter 3, Fig 3.2) was carried out by Ms Yee-Man Ching. The single fibre experiments carried out in Chapters 3, 4 and 5 were performed by Dr John Adcock, with my assistance.

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List of Abbreviations

4 α PDD:	4 α -phorbol 12,13-didecanoate
ACCP:	American College of Chest Physicians
AHR:	Airway Hyperresponsiveness
AI:	Adaptation Indices
AMP:	Adenosine Monophosphate
ATP:	Adenosine Triphosphate
AUC:	Area Under the Curve
BALF:	Bronchialveolar Lavage Fluid
CCIQ:	Chronic Cough Impact Questionnaire
CGRP:	Calcitonin Gene Related Peptide
CNS:	Central Nervous System
COPD:	Chronic Obstructive Pulmonary Disease
CQLQ:	Cough Quality of Life Questionnaire
CS:	Cigarette Smoke
CV:	Conduction Velocity
CVA:	Cough Variant Asthma
Dil:	DilC ₁₈ (3) (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribose Nucleic Acid
EET:	Epoxyeicosatrienoic acid
GPCR:	G Protein Coupled Receptor
GSK101:	GSK1016790a
i.p:	Intra-peritoneal
i.v:	Intra-vascular
IIAM:	International Institute for the Advancement of Medicine
LCQ:	Leicester Cough Questionnaire
mOsm:	milli-osmole
NMDA:	N-methyl-D-aspartate
NTS:	Nucleus Tractus Solitarius
OTC:	Over the Counter
OVLT:	Organum Vasculosum of the Lamina Terminalis
PAR:	Protein Activated Receptor
PCR:	Polymerase Chain Reaction
PGD ₂ :	Prostaglandin D ₂
PGE ₂ :	Prostaglandin E ₂

RAR:	Rapidly Adapting Receptor
RLN:	Recurrent Laryngeal Nerve
RNA:	Ribonucleic Acid
ROS:	Reactive Oxygen Species
RT:	Room Temperature
RTPCR:	Reverse Transcription Polymerase Chain Reaction
RTX:	Resiniferatoxin
S.E.M.	Standard Error of the Mean
SAR:	Slowly Adapting Receptor
SLN:	Superior Laryngeal Nerve
TRP:	Transient Receptor Potential
URTI:	Upper Respiratory Tract Infection
VGKC:	Voltage Gated Potassium Channel
VGSC:	Voltage Gated Sodium Channel
WHO:	World Health Organisation
WT:	Wild Type
$\alpha\beta$ -MeATP:	$\alpha\beta$ -MethyleneATP

Chapter 1:

Introduction

1. Introduction

1.1: Cough

Cough is a troublesome symptom, and is the most common reason for patients to visit a doctor in the UK (Schappert and Burt, 2006; Schappert and Rechtsteiner, 2011). Under normal circumstances, cough is a vital defensive reflex; protecting the airways from inhalation of foreign materials and harmful substances and also aids in immune defence (Irwin *et al.*, 1998; Fontana *et al.*, 1999; Widdicombe, 1995). However, in certain conditions which can occur in disease, the cough response can become excessive which can dramatically impact quality of life of sufferers. Despite this, there are currently no safe and effective treatments for cough.

Cough is a reflex response driven by sensory nerve fibres housed within the vagus nerve, which are tailored to detect changes in the physical and chemical environment (Brouns *et al.*, 2012). These fibres express a number of ion channels which detect and respond to a diverse range of stimuli, a number of which have been implicated in the cough response and can be activated by a number of exogenous and endogenous mediators. There is a large body of data outlining the role of the Transient Receptor Potential (TRP) family of ion channels in the cough reflex, where activation of two channels; TRPA1 and TRPV1 have been shown to cause cough in both animals and man (Wortley *et al.*, 2014; Andr  *et al.*, 2009; Birrell *et al.*, 2009; Grace *et al.*, 2012). Furthermore, an antagonist of the ionotropic purinoceptor P2X3 has been shown to inhibit the objective cough frequency in patients suffering from treatment resistant chronic cough (Abdulqawi *et al.*, 2014). Further investigation into the mechanism of action of ion channels present on the afferent arm of the cough reflex may help to increase our understanding of the cough reflex, and uncover potential novel therapeutics.

1.1.1: Physiology of the cough reflex

The cough reflex is a forced event used to clear the larynx, trachea and large bronchi from foreign material and secretions and prevent asphyxiation (Chung and Pavord, 2008). Physiologically, cough can be separated into three phases, beginning with a rapid deep inspiration, followed by a compressive phase where the intrathoracic pressure builds up following closure of the glottis. The reflex ends with an expiratory phase; a rapid expulsion of air, which can reach 500mph (Bianco *et al.*, 1988), and the lungs and airways are cleared of irritant materials. This release of air results in a characteristic cough sound that all are familiar with (Smith, 2010). Cough itself can occur singly, or in bouts where repetitive coughs occur in succession (Nasra and Belvisi, 2009).

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1.1.2: Prevalence of cough

A persistent cough is currently the most common reason for patients to visit a doctor in the UK (Schappert and Burt, 2006; Schappert and Rechtsteiner, 2011). The clinical aetiology of cough can be defined as either acute (under 3 weeks in duration (Irwin *et al.*, 1998)), or chronic (over 8 weeks in duration). Acute cough occurs mainly as a result of viral or bacterial respiratory tract infections (Curley *et al.*, 1988; Irwin *et al.*, 1998) or viral infections (common cold) (Irwin *et al.*, 1990) and often resolves itself a few weeks following treatment and clearance of the infection (Irwin, 2006). Chronic cough is a common symptom of the inflammatory diseases asthma and Chronic Obstructive Pulmonary Disease (COPD) (Fuller and Choudry, 1987; Irwin *et al.*, 1998; Morice *et al.*, 2007), where presenting with chronic cough often is a key feature leading to diagnosis of both diseases (McGarvey *et al.*, 2008, Vestbo *et al.*, 2013). Chronic cough can also arise in pulmonary fibrosis (Irwin *et al.*, 1998), Post Nasal Drip (PND), Gastro-Esophageal Reflux Disease (GERD), as a side effect of medications such as ACE inhibitors (Sesoko and Kaneko, 1985; Laloo *et al.*, 1996; Fahim *et al.*, 2011; Faruqi *et al.*, 2014), or it can also be idiopathic in origin. Idiopathic chronic cough can be triggered by normally innocuous stimuli and lead to unproductive bouts of coughing, which are not consciously suppressed (Pratter, 2006). Epidemiological studies have revealed that chronic cough can affect up to 40% of the population at any one time (Cullinan, 1992; Janson *et al.*, 2001; Morice *et al.*, 2001). This disease can affect patients for months or years at a time ultimately impacting on their quality of life (French, 1998). Long term excessive coughing can lead to excessive sweating, cough syncope, sleep disturbance, nausea and urinary incontinence (Irwin and Curley, 1991; French, 1998; French *et al.*, 2002). In addition, chronic cough can also have a negative impact on social relationships with friends and family (Brignall *et al.*, 2008), and many patients suffer from high levels of anxiety and depression (McGarvey and Morice, 2006). Despite this, success rates of treating chronic cough have been reported to be as low as 58% (Haque *et al.*, 2005).

1.2. Causes of Chronic Cough

1.2.1: Asthma

Asthma is a chronic inflammatory disease of the airways characterised by bronchoconstriction, which is mostly reversible, airway hyperresponsiveness (AHR) and symptoms such as dyspnoea, chest tightness, wheezing and cough (Lemanske and Busse, 2003). The World Health Organisation (WHO) has estimated that over 300 million people worldwide suffer from asthma, and the prevalence and mortality are increasing (Lemanske and Busse, 2003; Bateman *et al.*, 2008). Asthma is often associated with allergy, when an individual is exposed to an allergen, this leads to the production of allergen specific Immunoglobulin E (IgE). When

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the individual is re-exposed to the allergen, this produces an asthmatic response, which leads to bronchoconstriction, AHR and an inflammatory response involving Th2 cells, eosinophils, mast cells and CD4⁺ cells (Barnes, 2008).

Almost all asthmatic patients present with cough as a symptom (Niimi, 2011), and the symptom of cough is key in the management and diagnosis of asthma (Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2007). In addition, some asthmatic patients suffer from cough variant asthma (CVA) (Corrao *et al.*, 1979). Here, patients present with cough as the predominant symptom, and classical symptoms of asthma such as wheeze, chest tightness and apnoea may be absent (Morice, 2004). Patients are often treated successfully with asthma therapeutics (Dicpinigaitis, 2006). Cough as a symptom was reported to have the greatest impact on the quality of life of patients, worse than both wheeze and sleep disturbance (Osman *et al.*, 2001). Furthermore, a 9 year study indicated that the worsening of cough has been shown to have the highest predictive weight for a poor outcome in asthma (de Marco *et al.*, 2006). The cough rate of asthmatics has been shown to be significantly greater than for healthy volunteers (Smith, 2010).

Asthma is associated with eosinophilic inflammation, which is demonstrated by increased levels in the sputum and from airway biopsies (Smith, 2010). In the majority of cases, cough responds well to anti-inflammatory agents (Dicpinigaitis, 2006) which reduce eosinophilic counts, suggesting that this eosinophilic inflammation may help to promote the increased cough response seen in asthma, and could affect airway sensory nerves which drive the cough response (Smith, 2010). Furthermore, there are also increased amounts of Prostaglandin E₂ (PGE₂) (Chaudhuri *et al.*, 2004) and bradykinin (Abe *et al.*, 1967) in the airways of asthmatic patients, which alongside adding to the inflammatory response, have also been shown to cause a tussive response themselves in both animals and humans (Choudry *et al.*, 1989; Maher *et al.*, 2009; Grace *et al.*, 2012). ATP, which is released during inflammation (Adriaensen *et al.*, 2004), has also been shown to be increased in the BAL of mice following allergen challenge compared to saline controls (Idzko *et al.*, 2007), and asthmatics have been shown to experience bronchoconstriction, cough and dyspnea following aerosolised ATP challenge (Basoglu *et al.*, 2005). Anti-inflammatory agents have been shown to successfully treat cough in CVA (Pavord, 2004) and the majority of chronic cough in asthmatics, suggesting that there is potentially a causative effect between inflammation and chronic cough in asthma.

1.2.2: COPD

COPD is associated with chronic airway inflammation and encompasses a number of pathologies including chronic bronchitis, bronchiolitis and emphysema (Bateman *et al.*, 2008). This disease is a global health problem and it is estimated that it will be the third leading cause

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of death worldwide by 2020 (Vestbo *et al.*, 2013). COPD is characterised by airflow limitation, which is both progressive and not fully reversible and persistent inflammation which can lead to functional changes in the airways from repeated tissue injury and repair (Di Stefano *et al.*, 2009). The chronic airflow limitation in COPD can be caused by a number of factors including small airways disease caused by obstructive bronchiolitis and emphysema, along with parenchymal destruction. Chronic inflammation can also lead to the narrowing of the small airways (Vestbo *et al.*, 2013). The key risk factor for the development of COPD is cigarette smoking (Sethi and Rochester, 2000), where chronic cigarette smoke exposure leads to a positive feedback loop of inflammation (Barnes, 2008). The Lung Health Survey showed that patients with COPD exhibiting chronic cough had a greater number of pack years smoking, a worse lung function and are more likely to be current smokers (Kanner *et al.*, 1999). Despite this, stopping smoking reduced the prevalence of chronic cough by over 80% after 5 years (Kanner *et al.*, 1999).

Chronic cough is one of the first complaints that patients with COPD present with and it is the key to diagnosis (Vestbo *et al.*, 2013). A survey uncovered that cough was experienced by 70% of COPD sufferers, and was reported to occur on a daily basis in 40% (Rennard *et al.*, 2002). Similarly to asthma, a defining feature of COPD is lung inflammation characterised by macrophages, neutrophils and CD8⁺ T-lymphocytes (Keatings *et al.*, 1996; Keatings *et al.*, 1997). Unlike asthma, the abnormal inflammation seen is resistant to anti-inflammatory agents such as glucocorticoids (Barnes, 2013).

COPD is associated with substantial airway inflammation; the bronchoalveolar lavage fluid (BALF), sputum and exhaled breath condensates of patients show an increase in inflammatory mediators and cellular components of inflammatory disease (Bhowmik *et al.*, 2000; Gompertz *et al.*, 2001; Biernacki *et al.*, 2003; Montuschi *et al.*, 2003), and this may help to contribute to the enhanced cough response seen. Furthermore, similarly to asthma, some mediators such as prostaglandins are known to cause cough themselves (Choudry *et al.*, 1989; Maher *et al.*, 2009), and others, such as tachykinins, are indirectly involved in the cough response (Joos *et al.*, 2003). ATP levels have also been shown to be increased in COPD (Mohnesin *et al.*, 2006; Mortaz *et al.*, 2010) and a recent study has indicated that ATP induced more dyspnea, cough and throat irritation in patients with COPD following aerosolised ATP (Basoglu *et al.*, 2015). Chronic cough in COPD can also occur due to the mechanical stimulation of excessive mucus production, where an accumulation of mucus is associated with disease progression (Hogg *et al.*, 2004), and epidemiological studies have shown that chronic cough and mucus secretion lead to a decline in lung function (Løkke *et al.*, 2006).

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Although normal clinical practice would be to target the underlying cause of chronic cough (Irwin *et al.*, 2006), in the case of COPD, unlike asthma, this is not possible as the disease is progressive and anti-inflammatory agents do not show any efficacy, and therefore specific targeted treatments may show greater efficacy in treatment of chronic cough in COPD.

1.2.3: Unexplained Chronic Cough

Unexplained, or idiopathic, chronic cough, is the diagnosis given to patients who present with chronic cough with no known underlying disease cause (McGarvey *et al.*, 2013). Initial diagnosis of chronic cough involves systematic evaluation to outline a suspected underlying cause, after which most can be identified (Irwin *et al.*, 1990; Morice, 2004, Morice *et al.*, 2006; Shields *et al.*, 2008). However when no underlying cause is found, and the cough response is unresponsive to treatment, cough is defined as idiopathic, or unexplained and this can account for 18-42% of patients in specialist cough clinics (Polley *et al.*, 2008, Haque *et al.*, 2005). Clinical examination, chest radiography and spirometry of these patients is normal (Birring, 2011), and this condition is found predominantly in female patients (up to 80% in some cases) where cough begins around the menopause, suggesting a hormonal link (Birring *et al.*, 2003; Mund *et al.*, 2005).

Although the underlying cause is unknown, chronic cough has been associated with airway inflammation, where increased levels of cysteinyl leukotrienes, histamine, PGE₂ and Prostaglandin D₂ (PGD₂) are found in the airways of patients with chronic cough compared to healthy controls (Birring *et al.*, 2004). Furthermore, an increased number of mast cells have been found in the BALF (McGarvey *et al.*, 1999). Chronic dry coughing has also been linked with hypothyroidism in post-menopausal women, as idiopathic coughers were shown to have mild chronic lymphocytic airway inflammation which was associated with organ specific autoimmune disease (Birring *et al.*, 2003), and a subset of post-menopausal women have also been shown to have elevated CD4⁺ lymphocytes (Mund *et al.*, 2005). However, treatment of unexplained chronic cough with traditional anti-inflammatories has been shown to be unsuccessful, indicating that there are further mechanisms underlying this condition.

Similar to chronic cough associated with asthma and COPD, chronic coughers show an elevated response to capsaicin cough challenge (Sumner *et al.*, 2013; Ternesten-Hasséus *et al.*, 2013). It has therefore been proposed that chronic coughers experience airway sensory hyperreactivity syndrome (Chung, 2011; Millqvist, 2011) due to patients experiencing a lower threshold to common tussive stimuli with no known underlying cause. The mechanisms underlying this hypersensitivity of the cough reflex are currently unknown (Pratter, 2006; Chung, 2011; Millqvist, 2011).

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1.2.4: Current therapies

Other than medications that aim to treat the underlying cause of chronic cough, there is a lack of successful, specific targeted therapies for cough. In the UK, over £100 million a year is spent on antitussive therapies (Dicpinigaitis, 2011) and in the USA, this figure is \$3 billion (Footitt and Johnston, 2009). Antitussive medications account for large number of over the counter medications (OTCs) taken and in the USA, a survey taken revealed that over half of all preschool children had been given OTCs, and 67% of these comprised of antitussive medications (Kogan *et al.*, 1994). This is despite the fact that a recent systematic review of over 2100 participants failed to support the efficacy of OTCs for the treatment of cough (Schroeder and Fahey, 2002), and a study has revealed that cough syrup has no pharmacological effect and instead acts as a lubricant to coat the throat (Eccles, 2006). Recently the American College of Chest Physicians (ACCP) has begun to advise against the use of antitussives with children suffering from an URTI (Bolser, 2006). In addition some children's antitussive medications have been withdrawn from public use because of the lack of therapeutic potential and also the emergence of some life threatening side effects in children under 2 (Centers for Disease Control and Prevention, 2007; Vassilev *et al.*, 2009).

Currently, the only cough therapeutics that show any efficacy are those which act via the Central Nervous System (CNS) such as opiates. A clinical study in patients with chronic cough revealed that morphine sulphate was efficacious in treating patients who did not respond to other antitussive medications (Morice *et al.*, 2007). Codeine is currently the reference antitussive of choice, despite a study indicating that codeine was no more effective in treating cough in patients with COPD than placebo (Smith *et al.*, 2006). Furthermore, opiates have wide ranging, potentially dangerous side effects such as respiratory depression, sedation and gastro-intestinal problems (Belvisi and Geppetti, 2004), revealing an urgent need for new, safe and effective medications for chronic cough. Peripherally acting drugs, which target the afferent arm of the airway sensory nerve pathway and receptors and ion channels present on the vagus nerve could show greater specificity and efficacy. These could act through reducing the efficacy of synaptic transmission, or by inhibiting action potential frequency which may show improved therapeutic potential with fewer side effects (Canning and Mori, 2011).

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1.3. The Cough Reflex and Airway Sensory Nerves

Sensory nerves within the airways detect changes in the physical and chemical environment and relay pulmonary sensory information to the CNS, which leads to reflex responses such as cough (Belvisi, 2002; Brouns *et al.*, 2012). Regardless of cause, animal studies have revealed that cough is initiated following activation of afferent fibres in the vagus nerve (Cranial nerve X) (Canning *et al.*, 2006b), which innervates other midline organs and tissues along with the airways (Standring, 2005). Vagal afferents have termini in and under the airway epithelium, and have cell bodies in the jugular and nodose ganglia, which project peripherally to the airways and centrally to the Nucleus Tractus Solitarius (NTS) in the brainstem (Belvisi, 2002). The jugular and nodose ganglia have different embryological origins; the jugular is neural crest derived and the nodose is epibranchial placode derived (Nasra and Belvisi, 2009), and as a result house different populations of nerve fibres. The cell bodies of the vagus then branch into the superior laryngeal nerve (SLN) and recurrent laryngeal nerves (RLN) which carry airway fibres to the trachea and bronchi (Belvisi, 2003; Canning, 2004).

The cough reflex is a neural circuit, which connects the periphery to the CNS. It differs to other respiratory and airway reflexes as it occurs in a discontinuous, threshold dependent manner (Canning and Mori, 2011). There are three phases to the cough reflex; the first involves the initiation of the reflex through activation of sensory nerves, the second occurs in the CNS where the reflex is processed and involves a complex neuronal network and thirdly, efferent motor neurons are stimulated leading to cough (Pacheco, 2014) (**Figure 1.1**).

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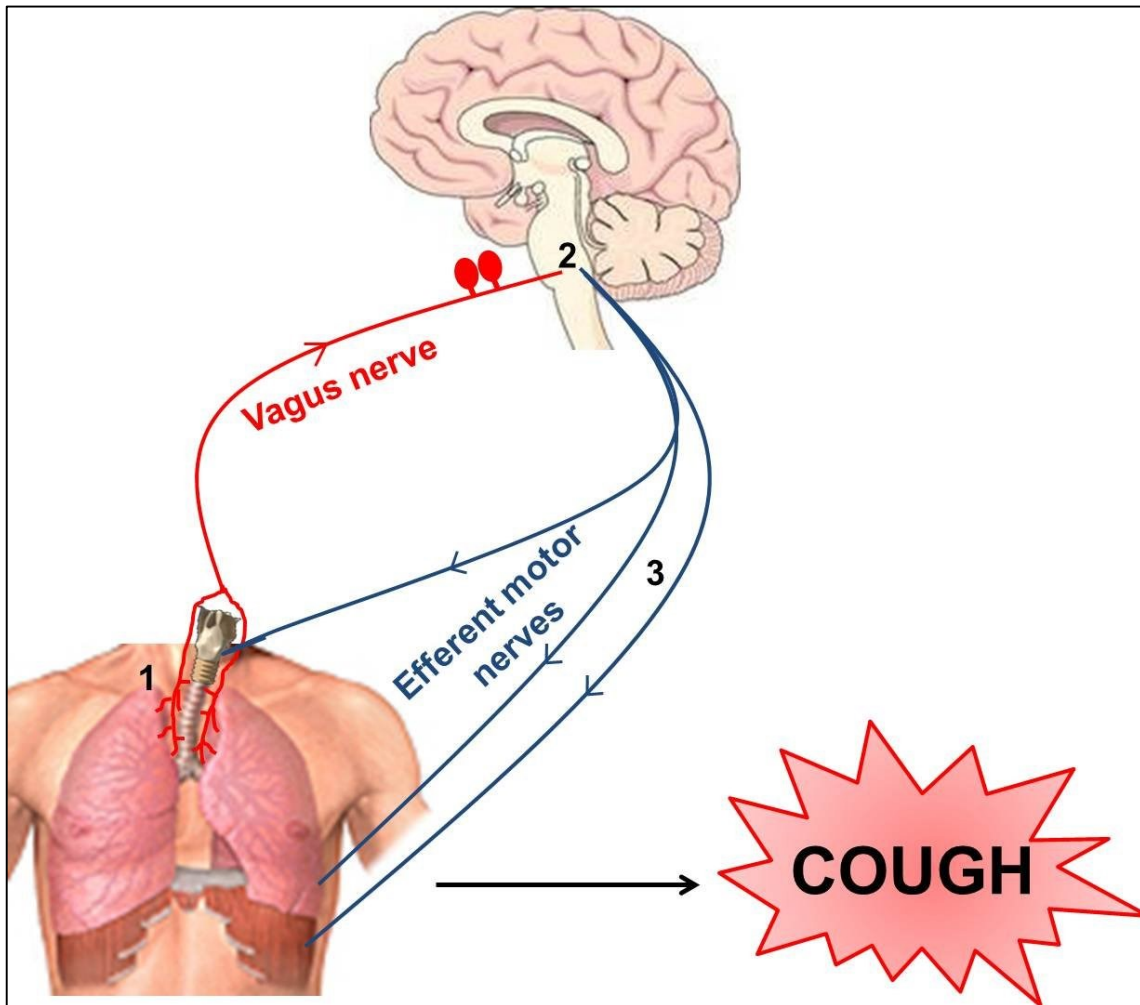


Figure 1.1: The cough reflex

- 1: An exogenous (e.g. cigarette smoke) or endogenous (e.g. PGE_2) stimulus activates receptors on vagal sensory nerve endings located in and under the epithelium. If this stimulus reaches a certain threshold, then an action potential is produced which is carried up the vagus nerve to the CNS.
- 2: The vagus nerve synapses with 2nd order interneurons within the Nucleus Tractus Solitarius (NTS) in the brainstem and the reflex is processed.
- 3: The signal is relayed via efferent motor neurons to the diaphragm, respiratory muscles and larynx to cause cough.

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Mechanical disturbance, inflammatory mediators, environmental stimuli, changes in pH, osmolarity, chemicals, and temperature can all activate airway sensory nerves and cause cough through activation of receptors present on the cell surface (Lowry *et al.*, 1988; McGarvey *et al.*, 1998; Wong *et al.*, 1999; Undem, 2004; Koskela *et al.*, 2005; Canning *et al.*, 2006a; Maher *et al.*, 2009; Belvisi *et al.*, 2011; Grace *et al.*, 2012). Activation of a receptor by a stimulus causes a change in ionic conductance across the membrane, depolarising the cell membrane. This initial depolarisation is known as a generator potential. This generator potential then has to reach a sufficient magnitude before an action potential can be produced (Brouns *et al.*, 2012). An action potential involves a rapid change in membrane potential which is propagated without decrement along the length of the cell and is triggered when the depolarisation is sufficient enough for the membrane potential to reach a critical value. The critical threshold of an action potential is dependent upon the gating potential of voltage gated sodium channels (VGSCs), which when opened trigger an influx of Na⁺. The normal resting potential of a membrane is -70mV, however following an influx of Na⁺ ions after the generator potential has reached a threshold value, the membrane becomes less negative and depolarises, which generates an action potential. This wave of depolarisation can then be propagated along the nerve fibre as action potentials to neuronal terminals within the CNS.

Once opened, the VGSCs become inactivated at a depolarised potential, which prevents further depolarisation of the cell membrane. Voltage gated potassium channels (VGKCs) then open, causing an efflux of positive K⁺ ions, and repolarisation of the membrane (**Figure 1.2**). The magnitude of an action potential is fixed and remains the same size and shape as it is propagated along the axon. Cough requires a sustained high frequency activation of afferent nerves for initiation (Canning and Mori, 2011), which leads to an urge to cough sensation preceding the reflex.

The mechanisms by which the afferent signals are processed in the CNS and cause cough are complex and not fully understood (Fong *et al.*, 2004). Anatomical and functional studies have revealed that the vagal afferents synapse on 2nd order interneurons in the NTS in the dorsomedial region of the medulla (Mazzone and Canning, 2002; Kubin *et al.*, 2006). Furthermore, it is thought that the region of CNS involved in processing the reflex is likely to be linked to the respiratory centre as a change in breathing pattern is an integral part of the cough response (Karlsson and Fuller, 1999). The major neurotransmitter involved in the cough reflex is likely to be glutamate as physiological and pharmacological investigations have revealed that N-methyl-D-aspartate (NMDA) glutamate receptors play a role in the central synapses of vagal nerves (Mutolo *et al.*, 2007, 2010; Canning, 2009, 2011).

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However interestingly, the cough reflex is under some level of conscious control, as coughs can be produced without stimulation, the cough reflex can be consciously enhanced, and cough can also be suppressed under certain circumstances (Canning and Mori, 2011).

Finally, the signal is sent via respiratory bulbospinal pre-motor neurons (I-Aug and E-Aug) to inspiratory and expiratory laryngeal motor neurons which cause a large contraction of the abdominal muscles and larynx which expels air and causes the characteristic cough sound (Bongianni *et al.*, 1998; Haji *et al.*, 2008).

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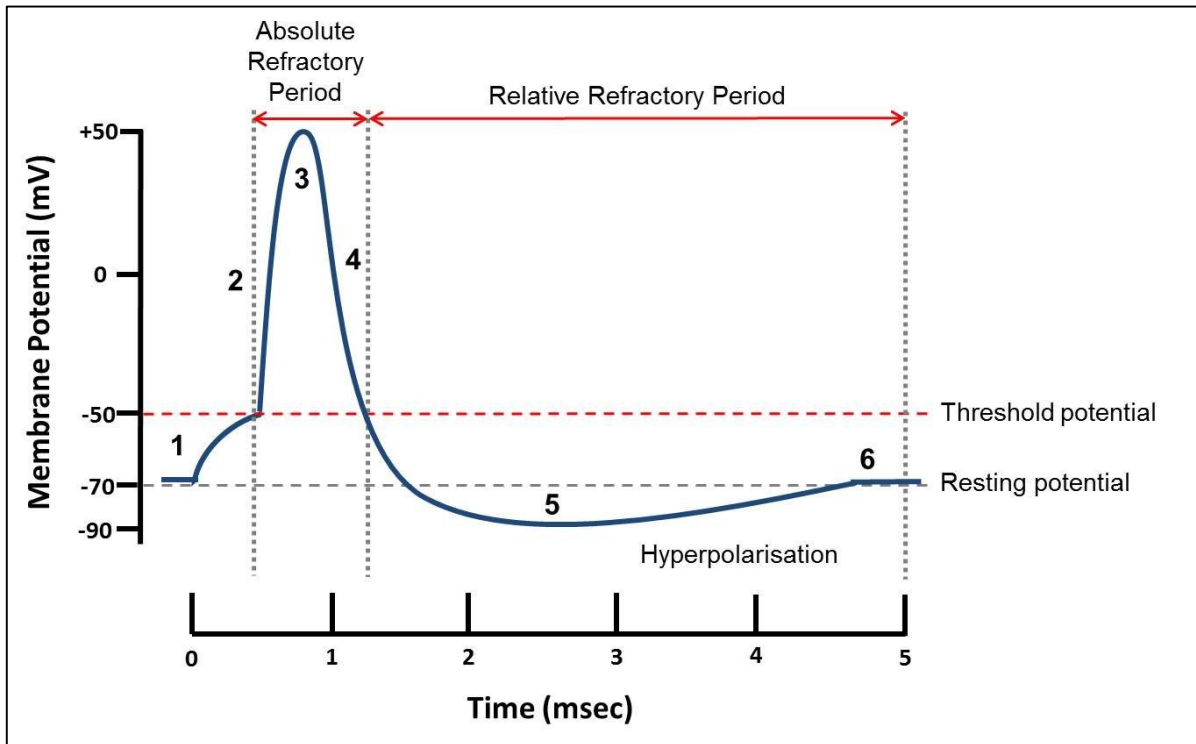


Figure 1.2: The Action Potential

1. The nerve cell is at a resting state, maintained by sodium and potassium pumps. A stimulus may be added to the cell but it has yet to reach the threshold potential
2. Threshold potential has been reached and VGSCs open leading to an influx of Na^+ ions into the cell, causing rapid depolarisation of the cell. At this point the cell is unable to fire another action potential regardless of how strong the stimulus. This stage is known as the absolute refractory period.
3. Action potential peak is reached and the VGSCs begin to inactivate, and the delayed rectifier VGKCs activate.
4. Activation of VGKCs causes a movement of K^+ out of the cell, causing rapid repolarisation of the cell. During this latter part of the action potential the cell is now capable of firing a second action potential, however a stronger than normal stimulus is required. This is known as the relative refractory period.
5. Closure of the VGKC is also delayed, causing hyperpolarisation of the cell.
6. The cell returns to resting potential.

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1.3.1: Sensory nerves subtypes and cough

Sensory nerves can be activated by a wide variety of stimuli, and can broadly be categorised into mechanoreceptors which respond to mechanical forces in the lung including those caused by inflation and deflation during respiration, and chemoreceptors which respond to both exogenous chemicals and inflammatory mediators which may potentially pose a threat of tissue damage (Brouns *et al.*, 2012). According to current understanding, there are five different subtypes of sensory nerve receptor within the airways; three of which are mechanically sensitive (and are all A δ -fibres, as in transmit action potentials in the A δ range); the rapidly adapting stretch receptors (RARs), slowly adapting stretch receptors (SARs) and the cough receptors, and two which are more chemically sensitive; C-fibres (which are further sub-divided into pulmonary and bronchial and/or jugular and nodose derived fibres) and A δ nociceptors (**Figure 1.3**). These fibres can be characterised according to a large number of properties including adaptation indices, physiochemical sensitivity, neurochemistry, origin, myelination, sites of termination and conduction velocities (CVs) (Canning *et al.*, 2006b). The properties of these fibre types have been discerned using studies in animals, and translational studies are yet to be undertaken in human tissue, therefore findings should be interpreted with some caution.

Rapidly Adapting Receptors (RARs)

RARs, which are also known as classical RARs, irritant receptors and A δ -fibres (Adcock *et al.*, 2014; Adcock *et al.*, 2003), are so named because they adapt rapidly (cease action potential propagation) to a maintained mechanical stimulus (Sant'Ambrogio and Widdicombe, 2001). These fast conducting, myelinated fibres occur throughout the respiratory tract from the nose to bronchi, but are mostly found in the larger airways where are thought to play a defensive role and respond to stimuli such as lung hyperinflation/deflation and light touch alongside citric acid (Mortola *et al.*, 1975; Sant'Ambrogio *et al.*, 1978; Adcock *et al.*, 2003; Canning *et al.*, 2006a; Ricco *et al.*, 1996; Sant'Ambrogio and Widdicombe, 2001). RARs conduct action potentials in the A δ range, with conduction velocities of over 3m/s (Canning *et al.*, 2006b). The cell bodies of RARs are thought to be mostly housed in the nodose ganglia, (Ricco *et al.*, 1996; Canning *et al.*, 2006b) and the receptors are not thought to be directly activated by chemical stimuli such as bradykinin and capsaicin, although these stimuli can indirectly activate these fibres following mucus production and bronchoconstriction (Widdicombe, 2003). However, recent work has suggested that a subset of RAR A δ fibres respond to capsaicin regardless of conduction velocity (Adcock *et al.*, 2014). RARs are capable of causing a cough response even under anaesthesia, and therefore thought to

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provide the defensive cough reflex which clears lungs of harmful particles (Nasra and Belvisi, 2009; Dicipinigaitis *et al.*, 2014).

Slowly Adapting Receptors (SARs)

SARs are also fast conducting, nodose derived myelinated fibres, conducting action potentials at approximately 18m/s (Canning *et al.*, 2006a), but in comparison to RARs they adapt more slowly to a maintained stimulus. They are also less sensitive than RARs to sustained lung inflation (Schelegle and Green, 2001). Termini are found in and within the smooth muscle of the airways (Bartlett *et al.*, 1976), and the receptors are involved in tidal breathing. SARs are especially important in the Hering Breuer reflex, a stretch reflex stimulated by vagal fibres that when stimulated causes the cessation of inspiration and the start of expiration (Schelegle, 2003). SARs are not thought to play a direct effect in the cough response.

Cough Receptors

The 'cough receptor' was first described by Canning and colleagues in 2004 as a fibre type similar to RARs in that it is derived from the nodose, and found in the larynx, trachea and bronchi. Additionally, these receptors respond to acid, pH and low threshold mechanical stimulation including punctate light touch, and are unresponsive to capsaicin, bradykinin and hypertonic saline (Canning, 2004; Mazzone, 2004). However, the cough receptors are more slowly conducting than RARs (transmit action potentials at ~5m/s) and are insensitive to stretch, pressure and bronchoconstriction which do activate RARs (Canning, 2004; Mazzone, 2004). However, as all research into this fibre has been carried out in animals, the relevance of this fibre type to the human cough response is yet to be determined (Canning, 2004; Mazzone, 2004; Nasra and Belvisi, 2009). Much of the characterisation of the A δ -fibres has been carried out *in vitro*.

A δ nociceptors

A δ nociceptors are fast conducting myelinated fibres which conduct action potentials ~4-6m/s with termini in the intra and extrapulmonary airways (Canning *et al.*, 2006a). They differ from RARs as they are less responsive to mechanical stimulation, originate in the jugular ganglia and express TRPV1, and so as a result they are responsive to capsaicin and PGE₂ (Riccio *et al.*, 1996; Kajekar *et al.*, 1999; Yu, 2005). Although these fibres are similar pharmacologically to C-fibres, their effect on cough has not yet been fully elucidated.

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C-fibres

C-fibres are slower conducting, unmyelinated fibres which transmit action potentials at around ~1m/s (Fox *et al.*, 1993; Riccio *et al.*, 1996; Mazzone, 2004; Canning *et al.*, 2006a). This fibre type make up the majority of afferent nerves which innervate the airways (Coleridge and Coleridge 1984, Lee and Pisarri 2001), and can originate from both the nodose and jugular ganglia. C-fibres innervate the epithelium and other structures within the airway wall in both intrapulmonary and extrapulmonary airways. The central reflex induced following activation of C-fibres is blocked by anaesthesia and they are therefore thought to be responsible for the conscious perception of airway irritation (Mazzone, 2004).

C-fibre endings, although responsive to mechanical stimuli, have a much higher threshold of activation than RARs/SARs (Coleridge and Coleridge, 1984; Ho *et al.*, 2001). They are however activated by a large number of chemical stimuli including agonists of the TRPV1 (e.g. capsaicin) and TRPA1 (e.g. acrolein) ion channels, along with endogenous stimuli such as PGE₂ and bradykinin, which are capable of causing a cough response in both animal models and man (Coleridge and Coleridge, 1984; Lalloo *et al.*, 1995; Canning, 2004; Dicipinigitis and Alva, 2005; Birrell *et al.*, 2009; Grace *et al.*, 2012; Dicipinigitis *et al.*, 2014; Adcock *et al.*, 2014). The expression of both TRPV1 and TRPA1 receptors have been confirmed in animal C fibres using single cell polymerase chain reaction (PCR) of airway ganglia (Nassenstein *et al.*, 2008; Brozmanova *et al.*, 2012), and furthermore TRPV1 has been shown to be expressed on human nerves (Groneberg *et al.*, 2004). The activation of C fibres by these chemical stimuli is a direct effect as the fibres are not responsive to changes in airway pressure and bronchoconstriction (Coleridge and Coleridge, 1984; Undem, 2004). However, this may differ on non C fibres.

C fibres can be further differentiated from A δ nociceptors and RARs as they are capable of synthesising neuropeptides which are transported to the central and peripheral nerve termini (Lundberg *et al.*, 1985; Baluk *et al.*, 1992; Hunter and Undem, 1999; Myers *et al.*, 2002) and can evoke reflex responses such as bronchoconstriction and mucus production (Nasra and Belvisi, 2009). However although this has been confirmed to occur in guinea pig and murine airways, it is not yet determined if this occurs in human airways.

There are two distinct populations of C fibres, termed 'bronchial' and 'pulmonary' C fibres according to their sites of termination. Bronchial C fibres are thought to innervate the extrapulmonary airways, are mostly derived from the jugular ganglia and are stimulated by chemicals in the bronchial and systemic circulation (Coleridge and Coleridge, 1984). These fibres also express neurokinins such as substance P and calcitonin gene-related peptide

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(CGRP) and is thought that these fibres are responsible for mediating the cough response because of their position within the airways (Undem, 2002a). In contrast, pulmonary C fibres are thought to innervate the lower airways, mostly do not express substance P or CGRP (Coleridge and Coleridge, 1984; Carr and Undem, 2003; Undem, 2004) and have been shown to inhibit cough in animal models (Tatar *et al.*, 1994; Widdicombe and Undem, 2002; Chou *et al.*, 2008). This inhibition is thought to be through inhibition of the respiratory centres in the CNS which cause cough rather than a direct inhibition of the cough reflex (Karlsson and Fuller, 1999).

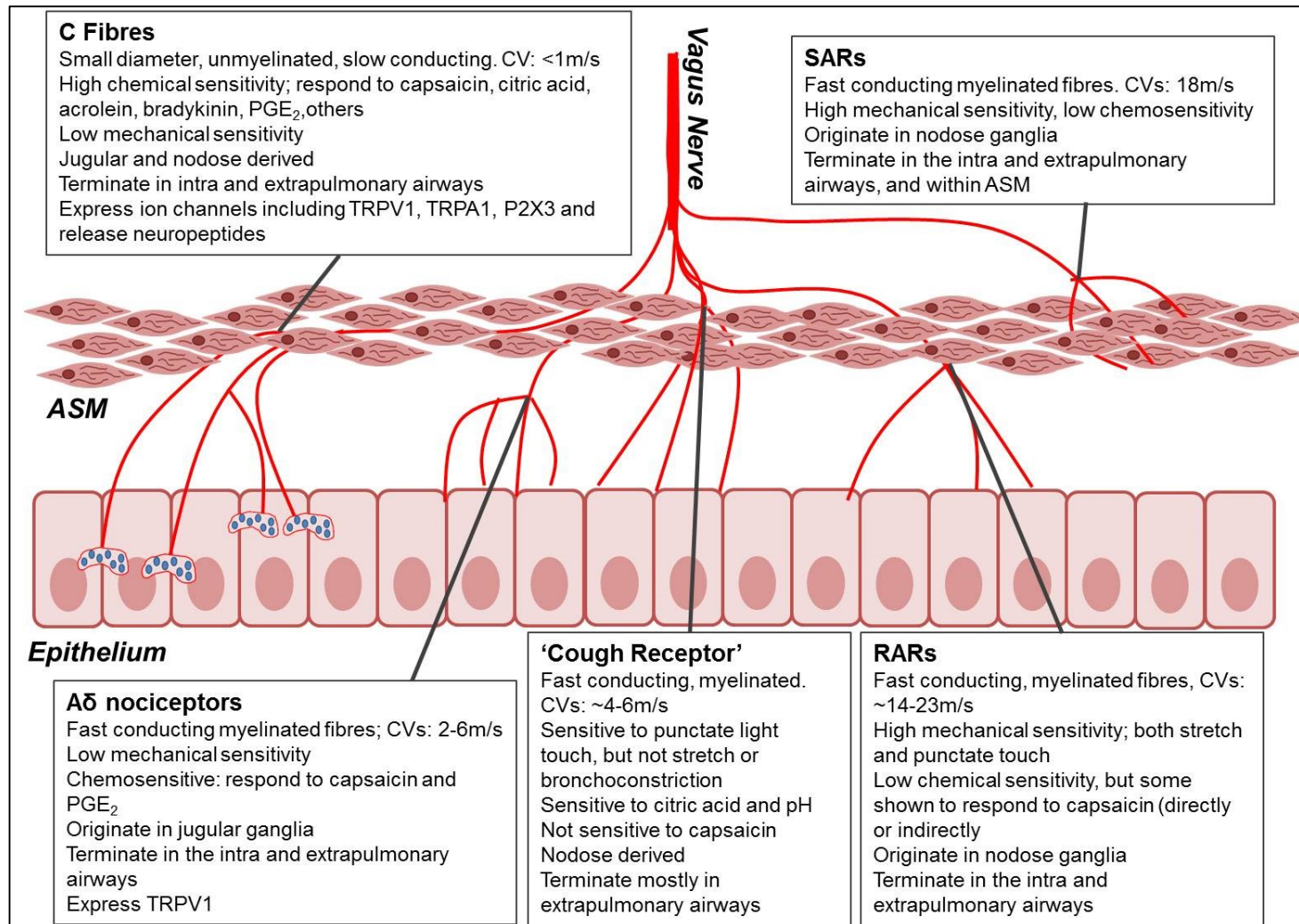


Figure 1.3: Summary of different vagal nerve fibre subtype properties in the airways

Information from (Sant'Ambrogio et al., 1978; Riccio, Kummer, et al., 1996; Widdicombe, 2003; Canning, 2004; Canning et al., 2006a; Nasra and Belvisi, 2009; Schelegle, 2003; Adcock et al., 2014)

1.4 Assessment of the cough reflex

1.4.1: Animal Models of Cough

The neural pathways, physiology and pharmacology of the cough reflex have been investigated using animal models in a number of species including guinea pigs, dogs, cats, rabbits, mice and rats (Gardiner and Browne, 1984; Hanáček *et al.*, 1984; Kamei *et al.*, 1993; Tatar *et al.*, 1994; Patel *et al.*, 2003). However, the anatomy and physiology of the guinea pig airways is the most similar to man, the lungs are innervated similarly (Canning, 2006a), and guinea pigs show similar sensitivity to the tussive stimuli capsaicin and citric acid (Karlsson and Fuller, 1999). Therefore it is widely agreed that the guinea pig provides the most suitable model system (Belvisi and Hele, 2003). The guinea pig can therefore be used in a number of *in vitro* and *in vivo* systems to investigate the cough reflex pre-clinically, many of which are used in this thesis and are described in greater detail in **Chapter 2**. Briefly;

- Cells from guinea pig nodose and vagal ganglia can be isolated and investigated. By administering a retrograde tracer dye DiI C18(3),1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) to the airways, 12-14 days prior to analysis, airway specific neurons can be isolated (Undem, 2002a; Kwong *et al.*, 2008; Lieu *et al.*, 2012). Once isolated, the cells can be loaded with a Ca^{2+} specific dye and intracellular calcium levels monitored in the presence of agonists and antagonists to determine activation (Grace *et al.*, 2012; Dubuis *et al.*, 2013). The cells can also be used for target expression at the mRNA level and electrophysiological assessment (e.g. patch clamp) (Nassenstein *et al.*, 2008; Dubuis *et al.*, 2013).
- The vagus nerve can also be isolated and placed into a grease gap recording chamber, where compounds can be applied to one end of the nerve trunk and compound membrane depolarisation recorded as a measure of activation (Birrell *et al.*, 2008; Fox *et al.*, 1995; Grace and Belvisi, 2011). It has been shown that many compounds which induce depolarisation of the isolated nerves in this preparation also induce a cough response *in vivo* (Patel *et al.*, 2003; Maher *et al.*, 2009). Alongside guinea pig tissue, translational data can be acquired using donor human vagal tissue, which is a major advantage of this technique.
- *In vivo* electrophysiology can be utilised to investigate the effects of pro-tussive or anti-tussive compounds on action potential firing of airway specific afferent fibres (Adcock *et al.*, 2003; Canning, 2004). The fibre type is identified using a range of criteria (pattern of spontaneous activity, response to hyper-inflation or deflation, adaptation

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indices, response to capsaicin and citric acid and conduction velocity), and the effects of compounds can be investigated on nerve termini in the whole animal, making the system more physiologically relevant.

- Finally, the guinea pig can be used as a model system for studying cough responses. This involves placing guinea pigs into individual Perspex chambers, and the effects of aerosolised compounds on the cough reflex measured. Studies have indicated that cough responses in animals are very similar to those seen in man (Laude *et al.*, 1993; Lee, 2006; Birrell *et al.*, 2009).

1.4.2: Clinical Assessment of Cough

Use of the guinea pig model in the investigation of airway sensory nerves and cough has uncovered a great deal regarding the mechanisms and triggers for cough. In order to translate these findings to the clinic, three main methods exist which monitor cough, and also help to evaluate the efficacy of novel antitussives, outlined below.

Cough questionnaires

Cough questionnaires provide a subjective, qualitative measurement of the impact of cough severity on daily life (Bonvini *et al.*, 2015). Currently, there are 3 validated and comparable questionnaires in use which measure cough as their end point. The Leicester Cough Questionnaire (LCQ) contains 19 items in 3 domains consisting of physical, psychological and social attributes and utilises a 17 point Likert Response scale (Birring 2003). Responses have been shown to correlate with cough visual analogue scores, and cough frequency measured using objective cough counters (Birring *et al.*, 2003; Birring *et al.*, 2006). The remaining 2 questionnaires are the Cough Quality of Life Questionnaire (CQLQ), which has 28 items in 6 domains (Simpson, 1999; French *et al.*, 2002) and Chronic Cough Impact Questionnaire (CCIQ) (Baiardini *et al.*, 2005). Using a specialised scoring system, these questionnaires evaluate the physical, psychological and social impact of coughing and are comparable in ascertaining the severity and impact of the cough response. However, an obvious disadvantage of this method is that it is reliant upon patient recall and is subjective.

Ambulatory cough counting

Ambulatory cough counting allows a non-invasive objective cough count to be taken in a patient's natural environment (Smith, 2010). The patient wears a cough monitor, which is a discrete recording device which can be worn under clothing, and is equipped with a microphone which

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measures cough over a defined time period, for up to 24 hours (Smith and Woodcock, 2008; Smith, 2010; Kelsall *et al.*, 2011).

Cough monitors are currently not commercially available, however there are several in use in cough clinics. A well known and widely published monitor is the Leicester Cough Monitor, however this is yet to become fully automated (Birring *et al.*, 2008; McGuinness *et al.*, 2008). It has been shown to have a high sensitivity in both patients and healthy individuals (around 82-83%) (Yousaf *et al.*, 2013). Another cough monitor in use is the Hull Automated Cough Counter which uses neural based technology to recognize sounds (Barry *et al.*, 2006), however it is still in development. The Vitalojak (Vitalograph Limited, Buckinghamshire, UK) is a custom made digital recording device which records cough through a microphone placed on the chest wall and on clothing (Smith and Woodcock, 2008). This system removes silences and non cough sounds from the recording using a specialised compression algorithm, and no longer requires calibration (McGuinness *et al.*, 2012; Smith, 2010) and has been shown to be highly selective in measuring cough from patients with chronic cough, asthma, COPD, lung cancer and healthy controls (McGuinness *et al.*, 2012).

The key advantage of using this technique is that it is a more representative measure of the cough reflex as it is not carried out in a laboratory environment, and is not reliant upon patient recall.

Cough reflex sensitivity

A cough challenge to certain pro tussive compounds, commonly capsaicin and citric acid, can be carried out in a laboratory environment to assess cough sensitivity between different patient subgroups. Capsaicin is commonly used as it induces cough in a dose dependable, reproducible manner (Dicpinigaitis, 2006) and is also safe to use, with no reported adverse effects (Dicpinigaitis and Alva, 2005). Sensitivity is commonly measured using C2 and C5 parameters; the concentration of tussive stimulus which is required to reach 2 or 5 coughs (Doherty *et al.*, 2000; Dicpinigaitis, 2006; Morice *et al.*, 2007). This is both easy to perform (Morice *et al.*, 2007) and also produces reproducible results (Dicpinigaitis, 2006) and is achieved by patients inhaling single doubling concentrations of cough stimulus, most commonly capsaicin (Hilton *et al.*, 2013). In addition, a key advantage of this technique is that clinicians are able to discern differences in cough sensitivity across different diseases, including asthma, COPD and chronic cough with the caveat that capsaicin challenge will only detect changes in TRPV1 pathway sensitivity.

However, several disadvantages of the use of C2 and C5 parameters have recently come to light. When compared to ambulatory cough counts, the results found that sensitivity testing only weakly

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correlates (Birring *et al.*, 2006; Decalmer *et al.*, 2007; Hilton *et al.*, 2013). Instead it has been suggested to use non linear dose response modeling, which utilises the E_{Max} and ED_{50} from a capsaicin challenge instead of C2 and C5, and has been shown to be able to distinguish between disease subgroups and healthy controls, and also correlates well with ambulatory cough counts, and also cough studies in animals (Hilton *et al.*, 2013; Smith *et al.*, 2012).

1.5: Ion channels implicated in the cough reflex

Airway sensory nerves express a number of ion channels which are activated by and respond to a wide range of mechanical, chemical and thermal stimuli. When these ion channels encounter potentially noxious stimuli they act as cellular sensors, to induce protective mechanisms in the airways such as cough and bronchoconstriction. A role of a number of these ion channels that have been implicated in the cough reflex are outlined below.

1.5.1: P2X3 receptors

The ubiquitous signalling molecule Adenosine Triphosphate (ATP) is found in every cell in the body where it plays a critical role in cellular metabolism and energy provision. It is released from cells under both physiological and pathophysiological requirements and plays a role as a local regulator and mediator in disease (Pelleg and Schulman, 2002). ATP activates the ionotropic P2X receptors and the metabotropic P2Y receptors (Burnstock and Kennedy, 1985), and is known to activate airway sensory nerves (Brouns *et al.*, 2000) where receptors containing P2X3 subunits have been shown to play a crucial role (Burnstock, 2001; Jarvis, 2003; Ford *et al.*, 2006). P2X3 receptors are members of the purinergic P2X family of ion channels, of which there are 7 members, which function as homo or heterotrimers formed of proteins subunits encoded by genes for P2X1-P2X7 (North and Surprenant, 2000; Gever *et al.*, 2006). P2X receptors show preference for ATP over breakdown products such as Adenosine Monophosphate (AMP) (Burnstock and Verkhratsky, 2010). P2X3 forms homotrimeric P2X3 receptors (consisting of 3 P2X3 subunits) or heterotrimeric P2X2/P2X3 receptors (consisting of 2 P2X3 subunits and 1 P2X2 subunit) (Ford, 2012).

ATP and P2X3 have been implicated in a number of respiratory diseases, and ATP levels in the BALF of COPD and asthmatic patients has been shown to be enhanced (Mohsenin and Blackburn, 2006; Polosa and Holgate, 2006; Idzko *et al.*, 2007; Lommatzsch *et al.*, 2010; Mortaz *et al.*, 2010). Furthermore, a recent clinical study has outlined a possible role for receptors containing the P2X3 subunit in the pathogenesis of chronic cough (Abdulqawi *et al.*, 2014). In this study, a selective P2X3 antagonist AF-219 was shown to significantly inhibit daytime objective

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cough frequency in patients with chronic cough (Abdulqawi *et al.*, 2014). This is the first antagonist that targets the afferent arm of the reflex which has shown any efficacy, and indicates that ATP and P2X3 could play a role in the cough reflex.

Expression

Although the majority of P2X receptors are widely expressed in neuronal, epithelial, muscular and immune cells (Burnstock and Knight, 2004), P2X3 expression is much more limited and although it can be found in epithelial cells and enteric neurons, receptors are mostly localised on sensory neurons (Chen *et al.*, 1995; Lewis *et al.*, 1995; Vulchanova *et al.*, 1996; Jin *et al.*, 2004; Wang *et al.*, 2005; Burnstock, 2008). P2X3 is predominantly expressed in the airways on small to medium diameter C or A δ fibres originating from the jugular and nodose ganglia (Kwong *et al.*, 2008; Undem and Nassenstein, 2009).

Activation

As mentioned previously, P2X3 is predominantly activated by ATP and the ATP derivative $\alpha\beta$ -Methylene ATP ($\alpha\beta$ -MeATP) is a potent agonist. $\alpha\beta$ -MeATP is a more stable ligand selective for P2X1 and P2X3 containing receptors. For activation to occur, three molecules of ATP are bound to extracellular portions of open P2X channels (Jiang *et al.*, 2003). P2X3 receptors are rapidly desensitised, the channel opens milliseconds following activation, and deactivates within tens of milliseconds (North and Jarvis, 2013).

When activated, P2X3 can then cause a variety of effects within the lung (**Figure 1.3**). Administration of ATP in the guinea pig, although did not cause cough alone, did significantly enhance the number of coughs to citric acid, and this enhancement was inhibited following application of a P2X1/P2X3 inhibitor TNP-ATP (Kamei *et al.*, 2005). ATP has also been shown to activate sensory fibres from the nodose and jugular ganglia, which was blocked by the selective P2X3 antagonist A-317492 (Kwong *et al.*, 2008). In humans, ATP was shown to cause cough and bronchoconstriction in asthmatic subjects, smokers and patients with COPD (Basoglu *et al.*, 2005; Basoglu *et al.*, 2015), and aerosolised ATP has also been shown to cause bronchoconstriction in normal subjects (Pellegrino *et al.*, 1996). Taken together this would suggest a role for ATP and P2X3 in the activation of airway sensory nerves and cough (**Figure 1.4**).

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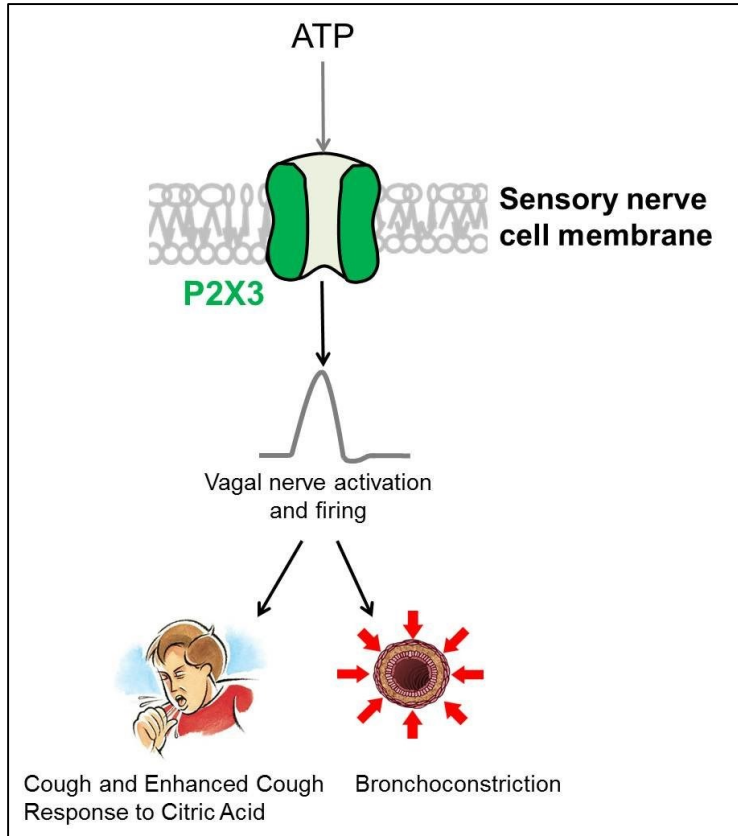


Figure 1.4: P2X3 activation

P2X3 present on airway sensory nerves is activated primarily by ATP (North and Jarvis, 2013; North and Jarvis 2010). This causes a variety of intracellular responses, including sensory nerve activation and firing in the guinea pig (Kwong et al., 2008), an increased tussive response to citric acid in guinea pigs (Kamei et al., 2005) and cough and bronchoconstriction in man (Pellegrino et al., 1996; Basoglu et al., 2005).

P2X3 antagonists as antitussives

To date, one small molecule inhibitor of P2X3 has advanced into clinic trials for chronic idiopathic cough. In a recent, placebo controlled clinical trial, AF-219 administered twice daily was shown to inhibit objective cough frequency by 75% in patients with chronic cough (Abdulqawi et al., 2014). There was also a significant improvement in all end point measurements compared to placebo (Abdulqawi et al., 2014). These results indicate that P2X3 may represent a promising new antitussive treatment.

1.5.2: Transient Receptor Potential family of ion channels

A number of members of the TRP family of ion channels have been implicated in the afferent arm of the cough reflex (reviewed in (Grace et al., 2013)). TRP channels are a superfamily of ion

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channels which respond to a variety of stimuli present in the cellular environment including changes in temperature, stretch, chemicals, oxidation, osmolarity and pH (Moran *et al.*, 2011; Picazo-Juárez *et al.*, 2011). These channels show a preference for Ca^{2+} and acquired their name from the original discovery in the *Drosophila* fly where they were shown to have a transient response to bright light (Montell and Rubin, 1989; Ramsey *et al.*, 2006; Caterina *et al.*, 1997). There are 28 members in 6 subfamilies; the caninocal TRPC, vanilloid TRPV, melastatin TRPM, ankyrin TRPA, polycystin TRPP and mucolipin TRPML which are categorised according to sequence homology and amino acid sequences (Clapham, 2003). All TRP channels share the same basic structure, with intracellular N and C termini, variable number of ankyrin repeats and 6 transmembrane domains with the cation pore present between domains 5 and 6 (Caterina *et al.*, 1997; Ramsey *et al.*, 2006). When TRP channels are activated, this causes an influx of cations and depolarises the membrane. If the stimulus reaches a critical threshold then an action potential is produced causing a wide range of cellular responses (Kaneko and Szallasi, 2014). Further investigation into the mechanism of action of these channels may lead to the development of novel therapeutics for the treatment of conditions such as chronic cough

Transient Receptor Potential Vanilloid 1 (TRPV1)

The polymodal ion channel TRPV1 has been heavily implicated in the cough response in both animals and man. It was the first TRP receptor to be identified as a key regulator of the cough reflex (Laloo 1995). Furthermore, the potent TRPV1 agonist capsaicin is one of the most commonly used tussive stimuli in the clinic (Szallasi and Blumberg, 1990; Caterina *et al.*, 1997).

Expression of TRPV1 in the airway

TRPV1 is highly expressed in a population of sensory neurons where it has been shown to mediate excitation and desensitisation to specific agonists (Szallasi and Blumberg, 1999). It was initially thought that expression of TRPV1 was confined to sensory neurons (Szallasi and Blumberg, 1999; Caterina *et al.*, 1997), but it has since been shown to be expressed in a variety of neuronal and non-neuronal tissues and organs, albeit at lower levels than that found in sensory ganglia (reviewed in (Tominaga and Tominaga, 2005)). Single cell PCR of airway ganglia has confirmed that TRPV1 mRNA is present in airway specific neurons (Nassenstein *et al.*, 2008; Lieu *et al.*, 2012). Selective antibodies for many of the TRP channel family are yet to be found, and therefore protein expression has not been confirmed. Instead, functional experiments involving changes in intracellular Ca^{2+} signal in ganglia, *and in vitro* and *in vivo* nerve depolarisation and isolated airway fibre firing studies have been utilised to determine the functional presence of

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TRPV1 on the vagus nerve. Utilising this approach, it has been shown that TRPV1 is functionally expressed on guinea pig airway jugular ganglia, where agonists cause intracellular Ca^{2+} flux, however, in normal healthy conditions, TRPV1 agonists have less of an effect on the nodose (Grace *et al.*, 2012; Hu *et al.*, 2014). In addition, capsaicin has been shown to induce firing of both C fibres and a population of RAR A δ fibres (Adcock *et al.*, 2014), suggesting the presence of the TRPV1 receptors on these fibres.

Activation of TRPV1

The TRPV1 ion channel is activated by a wide range of exogenous and endogenous mediators, including irritant chemicals, acid, noxious heat (temperatures above 43°C), and endogenous mediators such as anandamide and HPETE (Caterina *et al.*, 1997; Zygmunt *et al.*, 1999; Smart *et al.*, 2000; Jia *et al.*, 2002; Kagaya *et al.*, 2002; Moriyama *et al.*, 2005a; Jordt *et al.*, 2000; Zhou *et al.*, 2011). Both capsaicin and resiniferotoxin (RTX) directly open the channel, whereas other agonists such as low pH and increased temperature are thought to sensitise the channel and lower the activation threshold for opening as well as directly opening the channel (Caterina *et al.*, 1997; Trevisani *et al.*, 2007; Tominaga, 2008). TRPV1 can also be indirectly activated by agonists of G Protein Coupled Receptors (GPCRs), which bind to GPCRs and initiate a signalling cascade which activates TRPV1. These agonists including bradykinin (Grace *et al.*, 2012), PGE_2 (Moriyama *et al.*, 2005; Grace, *et al.*, 2012) and activators of the Protease Activated Receptors (PARs) (Amadesi *et al.*, 2004).

Activation of TRPV1 present on airway afferents causes a wide range of effects in the airways. It is well known that capsaicin causes a cough response in both animals and man (Fox *et al.*, 1995c; Laloo *et al.*, 1995; Caterina *et al.*, 1997; Nasra and Belvisi, 2009; Ricco *et al.*, 1996), and both direct agonists such as capsaicin and RTX and indirect agonists such as PGE_2 have been shown to functionally activate guinea pig and mouse vagal tissue (Fox *et al.*, 1995c; Maher *et al.*, 2009; Grace *et al.*, 2012; Birrell *et al.*, 2014). These agonists also activate donor human vagal tissue, thereby producing translational results (Birrell *et al.*, 2009; Birrell *et al.*, 2014; Usmani *et al.*, 2005). TRPV1 ligands have also been shown to cause firing of single afferent C fibres (Adcock, 2009; Adcock *et al.*, 2014) and a subset of A δ fibres (Adcock *et al.*, 2014). Other agonists which cause cough such as PGE_2 , citric acid, low pH and bradykinin do so through activation, at least partially, through the TRPV1 ion channel (Fuller and Choudry, 1987; Karlsson and Fuller, 1999; Doherty *et al.*, 2000; Morice *et al.*, 2007; Laude *et al.*, 1993; Laloo *et al.*, 1995; Morice *et al.*, 2001; Kollarik and Undem, 2002; Maher *et al.*, 2009; Grace *et al.*, 2012). Capsaicin is also capable of indirectly

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causing bronchoconstriction, via the release of sensory neuropeptides such as substance P and neurokinin A from C fibre terminals, which activates receptors on airway smooth muscle (Lalloo *et al.*, 1995; Lundberg *et al.*, 1985; Belvisi, 2002) and can could also activate mechanoreceptors (Figure 1.5).

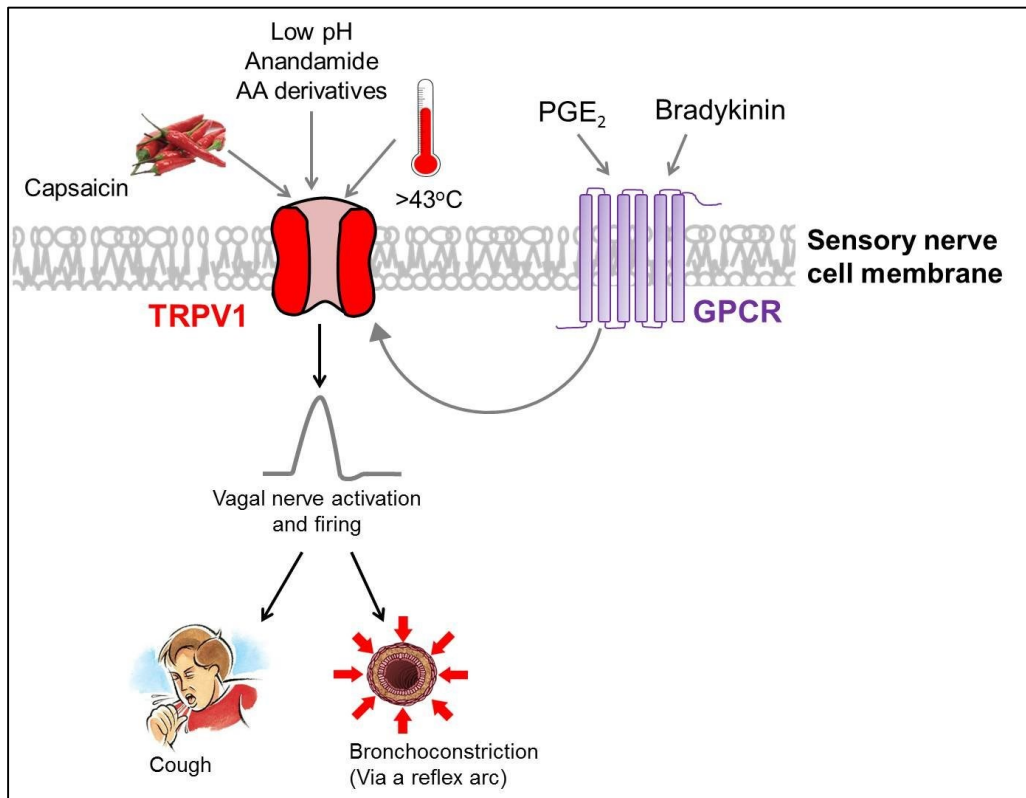


Figure 1.5: TRPV1 activation

Capsaicin is a potent activator of TRPV1 (Caterina *et al.*, 1997), along with low pH (Jordt *et al.*, 2000), the endocannabinoid eicosanoid derivative anandamide (Smart *et al.*, 2000), arachidonic acid derivatives (Zygmunt *et al.*, 1999; Kagaya *et al.*, 2002) and temperatures exceeding 43°C (Caterina *et al.*, 1997). TRPV1 activation also occurs downstream of activation of GPCRs by PGE₂ and Bradykinin (Grace *et al.*, 2012). Once activated, TRPV1 can cause several cellular effects which initiates following activation of both human and guinea pig vagal nerves (Maher *et al.*, 2009; Grace *et al.*, 2012; Birrell *et al.*, 2014). TRPV1 agonists have been shown to cause cough in both animal and humans (Fox *et al.*, 1993; Lalloo *et al.*, 1995; Caterina *et al.*, 1997; Nasra and Belvisi, 2009; Riccio, *et al.*, 1996) and bronchoconstriction as the result of parasympathetic activation and release of ACh (Raemdonck *et al.*, 2012) or through release of neuropeptides (Lalloo *et al.*, 1995; Lundberg *et al.*, 1985; Belvisi, 2002).

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TRPV1 in disease

TRPV1 has also been shown to play an important role in airway disease, where expression levels of the channel are altered. Animal models of disease have indicated that exposure to cigarette smoke; allergen and virus can lead to hypersensitivity of TRPV1 agonist inhalation (Karlsson *et al.*, 1991; Lewis *et al.*, 2007; Maher and Belvisi, 2010; Grace and Belvisi, 2011b; Lieu *et al.*, 2012). Following both cigarette smoke (CS) and allergen exposure, nodose ganglia from the guinea pig have been shown to become more responsive to the TRPV1 agonist capsaicin, therefore proposing that there is a phenotypic switch in receptor expression in disease (Wortley *et al.*, 2011; Lieu *et al.*, 2012). Furthermore, in a CS exposure model, guinea pig and human vagal tissue show enhanced responses to TRPV1 agonists (Wortley *et al.*, 2011). In addition, six TRPV1 SNPs associated with a higher risk of chronic cough have also been identified from two independent studies from 8 European countries (Smit *et al.*, 2012). It has also been established that there is an increased tussive response to capsaicin in a number of animal disease models (Karlsson *et al.*, 1991; Carr *et al.*, 2002; Myers *et al.*, 2002; Lewis *et al.*, 2007; Maher and Belvisi, 2010; Wortley *et al.*, 2011). In addition, patients with airway diseases such as COPD and idiopathic cough show an increased cough response to inhaled capsaicin (Choudry and Fuller, 1992; Groneberg, 2004; Nieto *et al.*, 2003; Prudon *et al.*, 2005; Ternesten-Hasséus *et al.*, 2006; Sumner *et al.*, 2013). Taken together with the fact that there is an increased amount of endogenous TRPV1 agonists present in the diseased airways (e.g. low pH, arachidonic acid derivatives, an increased temperature), further implicates TRPV1 in the cough response.

TRPV1 antagonists as potential antitussives

A role for TRPV1 in patients with chronic cough has been outlined as these patients exhibit an increased tussive response to capsaicin compared to healthy controls (Choudry and Fuller, 1992; Nieto *et al.*, 2003; Groneberg *et al.*, 2004; Prudon *et al.*, 2005; Ternesten-Hasséus *et al.*, 2006; Sumner *et al.*, 2013), and increased expression of TRPV1 has been demonstrated to occur in vagal nerves in patients with idiopathic cough (Groneberg *et al.*, 2004). Furthermore, TRP channels are chemically tractable targets and therefore there is an opportunity to develop effective therapies (Veldhuis *et al.*, 2015). Taken together this would suggest that TRPV1 is an attractive target for the treatment of chronic cough. However, traditionally, TRPV1 antagonists are known to cause hyperthermia and impaired perception of noxious heat which is a problem for potential antitussive compounds (Gavva *et al.*, 2007, 2008; Eid, 2011; Krarup *et al.*, 2011). Two recent clinic studies have utilised TRPV1 inhibitors which seem to have circumvented the issue of

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hyperthermia. The TRPV1 antagonist SB 705948 (Gunthorpe and Chizh, 2009) is rapidly absorbed with a long elimination life of 50-60 hours (Chizh and Sang, 2009). However, using both capsaicin cough challenge and 24 hour ambulatory cough monitoring, SB 705498 caused a very small shift in the capsaicin induced cough response curve, and did not affect spontaneous cough (Khalid *et al.*, 2014). However despite this lack of efficacy of the drug, it does not rule out a role for TRPV1 in disease related chronic cough given the lack of proof of target engagement in this study. A double blind, randomized, placebo controlled phase II clinical trial is currently being undertaken in patients with COPD with a novel highly potent TRPV1 antagonist XEN-DO501. The primary end point is objective cough measurement in patients with COPD, alongside a capsaicin challenge to ensure target engagement. This compound has previously undertaken Phase I studies which have shown it to be both well tolerated and safe, and has shown no issues with hyperthermia (Round *et al.*, 2011), Preclinical studies have shown that XEN-D0501 also inhibits capsaicin induced cough in naïve guinea pigs, and guinea pigs exposed to cigarette smoke (Wortley *et al.*, 2014).

Transient Receptor Potential Ankyrin 1: TRPA1

The ion channel TRPA1 is a Ca^{2+} permeable, non-selective cation channel with 14 ankyrin repeats in its amino terminus (Voets *et al.*, 2005). It was initially isolated from cultured fibroblasts (Jaquemar *et al.*, 1999) and is the only known mammalian member of the Ankyrin TRP family. Similarly to TRPV1, TRPA1 has also been implicated in the tussive response, where it is activated by a wide range of environmental irritants including cigarette smoke components such as acrolein and crotonaldehyde (Bautista *et al.*, 2006; Andr  and Campi, 2008a; Andr  *et al.*, 2009; Birrell *et al.*, 2009; Lin *et al.*, 2010; Volpi *et al.*, 2011). It is therefore thought to be the major mediator of peripheral irritant sensation as it is bound to and activated by a wide range of pungent natural compounds known to cause pain and inflammation (Macpherson *et al.*, 2005; Bautista *et al.*, 2013; Bandell *et al.*, 2004).

Expression

Similarly to TRPV1, the majority of TRPA1 expression is found in neuronal tissue, including spinal DRGs, nasal trigeminals and vagal airway neurons (Bandell *et al.*, 2004; Bautista, 2005; Nassenstein *et al.*, 2008; Jang *et al.*, 2012; Story *et al.*, 2003). Protein expression is not yet established, however, functional expression of TRPA1 has been determined in ganglia cells, where agonists of the TRPA1 channel induce Ca^{2+} flux in jugular, but not nodose, ganglia (Grace

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et al., 2012), and TRPA1 agonists also induce depolarisation of both animal and human vagal nerves *in vitro* (Birrell *et al.*, 2009; Grace *et al.*, 2012).

Activation

Covalent modification of N-terminal cysteines or lysines by electrophilic compounds is the major mode of activation of the TRPA1 channel (Bandell *et al.*, 2004; Hinman *et al.*, 2006; Banner *et al.*, 2011). TRPA1 was initially characterised as a thermal receptor activated by noxious cold (Story *et al.*, 2003), however it has since been shown that it can be activated by a diverse range of environmental and natural irritants such as mustard oil (Capasso *et al.*, 2012; Jordt *et al.*, 2004), cinnamaldehyde (Bandell *et al.*, 2004), and allicin (garlic) (Bautista *et al.*, 2006), tear gas, exhaust fumes, components of cigarette smoke (e.g. acrolein), formalin and $\alpha\beta$ -unsaturated aldehydes (Bandell *et al.*, 2004; Bautista *et al.*, 2006; McNamara *et al.*, 2007; Andr  *et al.*, 2008; Macpherson *et al.*, 2005; Bautista *et al.*, 2013; Dicipinigaitis *et al.*, 2014). Furthermore, TRPA1 is the target for many by-products of oxidative stress including Reactive Oxygen Species (ROS) and other electrophilic compounds including nitric oxide, hydrogen peroxide and hypochlorite (Bautista, 2005; Bessac and Jordt, 2008; Andersson *et al.*, 2008; Sawada *et al.*, 2008; Takahashi and Mori, 2011). TRPA1 is also indirectly activated by GPCR agonists such as PGE₂ and bradykinin (Grace *et al.*, 2012).

Activation of the channel leads to a wide range of effects in both animal and human tissue including activation of vagal bronchopulmonary C fibres (Bessac and Jordt, 2008; Nassenstein *et al.*, 2008; Taylor-Clark *et al.*, 2008), sensory nerve depolarisation (Birrell *et al.*, 2009; Grace *et al.*, 2012), cough (Andr  *et al.*, 2009; Birrell *et al.*, 2009; Grace *et al.*, 2012) and also bronchoconstriction, secondary to release of neuropeptides (Andre *et al.*, 2008) (**Figure 1.6**). Unlike TRPV1 ligands, the TRPA1 ligand acrolein was only capable of activating C fibres, and had no effect on A δ fibres in guinea pig airway single fibre firing (Adcock *et al.*, 2014).

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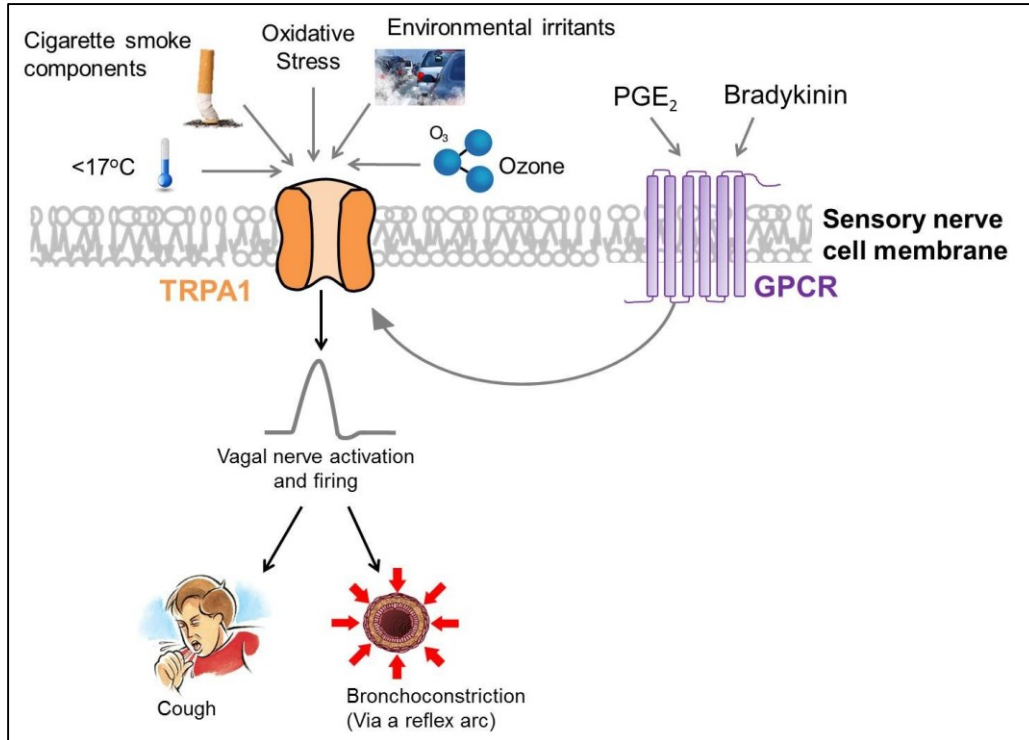


Figure 1.6: TRPA1 activation

TRPA1 is activated by a number of exogenous and endogenous ligands including low temperatures (below 17°C) (Story et al., 2003), cigarette smoke and components including acrolein (Andre et al., 2009; Bautista et al., 2006), oxidative stress (Bessac and Jordt, 2008), environmental irritants such as wood smoke (Shapiro et al., 2013) and ozone (Taylor Clark et al., 2010). It can also be indirectly activated by agonists of GPCRs such as PGE₂ and bradykinin (Grace et al., 2012). Downstream effects include sensory nerve firing in animals and man (Andre et al., 2009, Birrell et al., 2009), cough in both animals and man (Birrell et al., 2009, Andre et al., 2009, Grace et al., 2012) and bronchoconstriction via the release of neuropeptides (Andre et al., 2008).

TRPA1 in disease

Despite the fact that many components of cigarette smoke are known to activate TRPA1 (Bautista et al. 2006; Andr  et al., 2008; Andr  et al. 2009; Lin et al. 2010; Volpi et al. 2011) it is not yet known if expression levels of the channel are altered in disease. Furthermore, Smit et al have stated that there are no TRPA1 SNPs associating smoking or occupational exposure with cough (Smit et al., 2012). Unlike TRPV1 agonists, the tussive response to TRPA1 agonists has not yet been shown to be altered in disease states.

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However, chronic cough in disease states is associated with inflammation and the release of endogenous direct and indirect TRPA1 ligands such as ROS and PGE₂ (Grace *et al.*, 2011, Grace *et al.*, 2012), suggesting that TRPA1 may play a yet undiscovered role.

TRPA1 antagonists as antitussives

As a sensor of oxidative stress, alongside its role as a sensor of exogenous and endogenous respiratory irritants and the fact that agonists are capable of causing a cough response in animals and man makes TRPA1 a prospective therapeutic target for the treatment of chronic cough. Utilisation of a TRPA1 antagonist may also circumvent the temperature regulation safety concerns that are involved with TRPV1 antagonists.

There are several TRPA1 antagonists currently in pre-clinical development for the treatment of airway disease such as asthma, which could show potential therapeutic capability for the treatment of cough. Janssen has a TRPA1 antagonist currently in the preclinical stages, which has been suggested to have antitussive activity (Preti *et al.*, 2012). However, Glenmark pharmaceuticals are the only company to have a compound currently undergoing clinical studies in both healthy and asthmatic volunteers in an allergen challenge model (Preti *et al.*, 2012). GRC 17536 has already been shown to inhibit citric acid induced cough in the guinea pig (Mukhopadhyay *et al.*, 2014) and has recently been taken forward into a Phase II clinical trial to treat patients with chronic cough.

Transient Receptor Potential Vanilloid 4 (TRPV4)

TRPV4 was initially characterised as a sensor of osmotic stress, as mice devoid of the TRPV4 receptors show abnormalities with systemic osmotic stimuli (Strotmann *et al.*, 2000; Liedtke and Friedman, 2003; Suzuki *et al.*, 2003). The channel is a Ca²⁺ permeable polymodal receptor with 6 ankyrin repeats within the cytosolic N terminus (Nilius and Szallasi, 2014) activated by moderate temperatures.

Expression

TRPV4 is widely expressed in the airways, including in smooth muscle, alveolar wall, lung tissue, macrophages, epithelial cells and lung vessels (Jia *et al.*, 2004; Alvarez *et al.*, 2006; Dietrich *et al.*, 2006; Yang, 2006; Baxter *et al.*, 2014). There are also high levels of expression in the epithelial linings of trachea, bronchi and lower airways (Alvarez *et al.*, 2006; Fernández-Fernández *et al.*, 2008; Willette *et al.*, 2008). TRPV4 has also been shown to be expressed in neurons of both the

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central and peripheral nervous system including in DRG neurons (Grant *et al.*, 2007) and mRNA levels of TRPV4 have been found in pulmonary sensory neurons (Ni *et al.*, 2006).

Activation

TRPV4 is activated by moderate temperatures ($>24^{\circ}\text{C}$) and is therefore constitutively active at normal body temperature. The channel has been well characterised as both a mechanosensor and osmosensor (Nilius *et al.*, 2001), but there are also a number of exogenous and endogenous ligands which activate the channel. The small molecule activator GSK1016790a (Everaerts *et al.*, 2010; Thorneloe *et al.*, 2012) and synthetic phorbol esters such as 4 α -phorbol 12,13-didecanoate (4 α PDD) (Watanabe *et al.*, 2002) have been shown to activate the TRPV4 channel in both *in vivo* and *in vitro* systems. TRPV4 has also been well characterised as a receptor for hypotonic solution (Liedtke, *et al.*, 2000; Strotmann *et al.*, 2000; Jia *et al.*, 2004), and other endogenous ligands including arachidonic acid derivatives such as 5'6' epoxyeicosatrienoic acid (EET) (Watanabe *et al.*, 2003; Vriens *et al.*, 2005). Similarly to both TRPV1 and TRPA1, TRPV4 activation has been linked to activation of a GPCR. Stimulation of PAR₂ has been shown to sensitise TRPV4 expressed on DRG neurons and amplify responses to painful stimuli alongside causing neurogenic inflammation (Alessandri-Haber *et al.*, 2006; Grant *et al.*, 2007). PAR₂ activation has also been shown to stimulate TRPV4 channels directly, through receptor operated gating of TRPV4 (Poole *et al.*, 2013; Grace *et al.*, 2014) (**Figure 1.7**).

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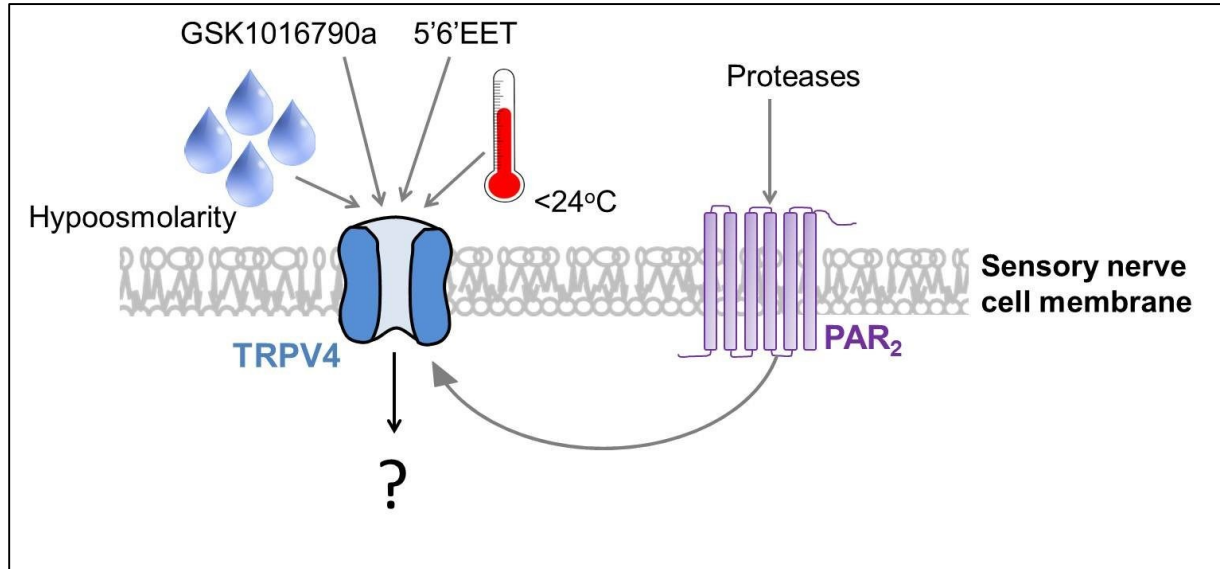


Figure 1.7: TRPV4 activation

TRPV4 is known to be activated by hypoosmolarity (Liedtke et al., 2000; Strotmann et al., 2000; Suzuki et al., 2003), small molecule activators such as GSK1016790a (Thorneloe et al., 2008), arachidonic acid derivatives such as 5'6'epoxyeicosatrienoic acid (Watanabe et al., 2003) and temperatures above 24°C (Nilius and Szallasi, 2014). TRPV4 can also be indirectly activated by agonists of PAR₂ (Poole et al., 2013). Downstream effects in airway sensory nerves are yet to be elucidated.

Role of TRPV4 in airway sensory nerves and cough

Despite the abundance of literature outlining the role of the TRPV1 and TRPA1 in sensory nerves and the cough reflex, relatively little is known about the role of TRPV4. The channel has already been shown to play a role in the airways, as agonists have been shown to cause contraction of airway smooth muscle via the release of cysteinyl leukotrienes (McAlexander et al., 2014), and 7 SNPs of the channel are associated with COPD pathogenesis (Zhu et al., 2009). As TRPV4 was originally characterised as a mechanosensor, this channel may therefore play a role in the more mechanically sensitive afferent airway fibres, rather than the chemosensitive C fibres which are activated by agonists of the TRPV1 and TRPA1 channels. Since antagonists of other ion channels expressed on airway sensory nerves such as TRPV1, TRPA1 and P2X3, have been shown to have promising therapeutic potential in the treatment of chronic cough, further understanding of the role of TRPV4 in airway sensory nerves and cough, may help to uncover a novel therapeutic target.

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1.6: Thesis plan

Hypothesis: Activation of TRPV4 causes activation of vagal sensory nerves and cough via the release of ATP and activation of P2X3.

In order to address this hypothesis, the aims of this thesis are;

- To pharmacologically assess the role of TRPV4 in airway sensory nerves and the cough reflex using selective agonists and antagonists of the channel and receptor deficient mice in a number of *in vivo* and *in vitro* systems. (**Chapter 3**).
- To investigate the possible role for endogenous mediators of TRPV4 in the activation of airway sensory nerves both *in vivo* and *in vitro* (**Chapter 4**)
- To investigate the potential mechanisms involved in TRPV4 induced activation of airway sensory nerves, and identify possible signalling pathways following TRPV4 activation involved in sensory nerve activation and cough (**Chapter 5**).

These aims will be achieved by;

- Using imaging of intracellular calcium flux in airway specific ganglia neurons to assess the effect of TRPV4 ligands in airway sensory nerves
- Using an *in vitro* isolated vagal nerve preparation which will allow direct investigation of the mechanism of activation of the airway afferents using relevant pharmacological tools
- Using *in vivo* electrophysiology to investigate single airway afferent firing using specific tool compounds
- Using an *in vivo* naïve guinea pig model to investigate the pro-tussive capabilities of TRPV4 ligands.
- Furthermore key experiments will be repeated using donor human vagus nerve tissue to enable translation of the findings in animal tissue.

Chapter 2:

Methodology

2. Methodology

2.1 Introduction

This chapter outlines the general methodology used for the techniques employed throughout my thesis. More detailed methods including experimental protocols and statistical analysis is included in the methods section within each chapter. A summary of all drugs, reagents and vehicles used is included in the Appendix.

2.2 Human and animal tissue

Male Dunkin Hartley guinea pigs and wild type (WT) male C57Bl/6j mice were purchased from Harlan (Bicester, UK) and housed in temperature controlled rooms (21°C) with food and water freely available for at least 7 days before commencing experiments. All work was approved by Imperial College London Ethics Review Committee, performed in accordance with the UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 and under Project Licence number 70/7212.

Donor human tissue unsuitable for transplant was obtained from the International Institute for the Advancement of Medicine (IIAM, NJ, USA). Ethics approval for human tissue use was obtained from the Royal Brompton & Harefield Trust

2.3 KO mouse breeding and genotyping

2.3.1: Genetically modified mouse breeding

Homozygous breeding pairs of mice genetically modified to disrupt the TRPV1 (*Trpv1*^{-/-}) and TRPA1 (*Trpa1*^{-/-}) genes were purchased from Jackson Laboratories (USA) (Caterina, 2000; Kwan *et al.*, 2006). Homozygous breeding pairs of genetically modified mice lacking the TRPV4 gene (*Trpv4*^{-/-}, RBRC No.01939) were obtained from Riken Bioresource Centre (Tsukuba, Japan) (Mizuno *et al.*, 2003; Suzuki *et al.*, 2003). Mice devoid of the Pannexin 1 gene (*Px1*^{-/-}) were a kind gift from Dr. Vishva Dixit, (Genentech, US) (Bargiotas *et al.*, 2011).

All mice were bred on a C57Bl/6 background, were viable and fertile and breeding colonies maintained at Imperial College London. Colonies of wild-type (WT) C57Bl/6 mice were bred alongside the genetically modified mice to be used as controls. Regular genotyping was undertaken to ensure the continuation of gene disruption in each generation.

2. Methodology

2.3.1: Genotyping and PCR

DNA extraction was performed on tail tips taken from genetically modified mice using a DNA extraction kit ('Extracta DNA prep for PCR', Quanta Biosciences, Gaithersburg USA). Tail tips (approximately 0.2cm) from each knockout mouse were removed and placed into 75µl of extraction buffer and heated at 95°C for 30 minutes. Once cooled to room temperature, 75µl of stabilisation buffer was added. The concentration of DNA could then be analysed using spectrophotometry at wavelengths of A260/A280 using GeneQuant RNA/DNA quantifier (Amersham Pharmacia, UK). The concentration of DNA was then adjusted to 10ng/ml using nuclease free water.

Once the DNA was extracted, a PCR was then undertaken to amplify the DNA sequence of interest between two primers. A reaction mixture totalling 25µl was made containing 50ng of extracted DNA (5µl), reaction buffer (5µl), 0.2mM dNTPs (1µl), 2mM MgCl₂ (2 µl), 1.25U Taq polymerase (0.2 µl), 10pmol forward and reverse primers of the gene target (1 µl) and the remainder of the reaction volume made up using nuclease free water. (PCR reagents were obtained from Promega, UK and primers from Invitrogen, UK). Samples were then heated to 94°C, following which 40 cycles of denaturing, annealing and extension were carried out, the exact temperature and duration of which was dependent upon the sequence of the primers used and the length of the expected DNA sequence (See **Table 2.1** for exact details of primers used). At the end of the 40 cycles, the samples were kept at 72°C for 10 minutes to ensure that all gene sequences were at full length, and then the reaction stopped by cooling the samples to 4°C for 5 minutes. The resulting PCR products were run on a 2% agarose gel (80 V for 1 hour) in TBE buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) alongside a relevant DNA ladder. The gel was then visualised under UV light and photographed

In the TRPV4, TRPV1 and Pannexin knockout mouse, an additional neomycin (Neo) primer was used. This was used to identify the knockout mice which had a neomycin cassette inserted into the target gene to produce the desired knockout genotype. The neomycin cassette gives resistance to neomycin allowing positive identification of knockout cells. Primer sequences were designed so that the WT amplicon would span the disrupted site of the desired gene which allowed for easier identification between wild type and knockout mice based on amplicon size.

2. Methodology

Table 2.1: Details of primer sequence for genotyping

TRPA1 WT Forward:	5'-TCA TCT GGG CAA CAA TGT CAC CTG CT-3'
TRPA1 WT Reverse:	5'-TCC TGC AAG GGT GAT TGC GTT GTC TA-3'
TRPA1 KO Forward:	5'-GAG CAT TAC TTA CTA GCA TCC TGC CGT GCC-3'
TRPA1 KO Reverse	5'-CCT CGA ATC GTG GAT CCA CTA GTT CTA GAT-3'
TRPV4 Forward:	5'-TGT TCG GGG TGG TTT GGC CAG GAT AT-3'
TRPV4 Reverse:	5'-GGT GAA CCA AAG GAC ACT TGC ATAG-3'
TRPV4 Neo:	5'-TGG ATT CGA CGC AGG TTC TC-3'
TRPV1 Forward:	5'-CCT GCT CAA CAT GCT CAT TG-3'
TRPV1 Reverse:	5'-TCC TCA TGC ACT TCA GGA AA-3'
TRPV1 Neo:	5'-CAC GAG ACT AGT GAG ACG TG-3'
Pannexin1 Forward:	5'-TGA CCA CAG ACA GCA CTT AAG-3'
Pannexin1 Reverse:	5'-CGT CTG AGA GCT CCC TGG CG-3'
Pannexin1 Neo:	5'-TCC GTT CAG TAC ATC CAC CTA CC-3'

2.4 Measurement of mRNA levels to assess gene expression

In order to determine if target genes were present in airway sensory nerves, real time PCR was undertaken in isolated whole ganglia from guinea pigs. The jugular and nodose ganglia provide the nerve fibres that make up the vagus nerve, which detects potential tussive stimuli within the lungs. Gene expression in the ganglia would suggest that (at least at the mRNA level) these genes are also present on the vagus nerve.

2.4.1: RNA extraction

The RNA extraction method was carried out according to the method previously described by McCluskie *et al* (McCluskie *et al.*, 2004). Guinea pig whole nodose and jugular ganglia were dissected from the animal, cleared of connective tissue and transferred to two separate autoclaved 2ml eppendorf tubes. The eppendorfs were autoclaved to ensure that no active ribonucleases were present which could cause the catalytic degradation of RNA. TriReagent (1ml,

2. Methodology

Sigma), was then added to each eppendorf and mixed, following which the samples were centrifuged (15,000g for 15min at 4°C) to remove any insoluble material such as cell membranes. The supernatant (which contains RNA, DNA and protein) was then transferred into a new eppendorf and left to stand at room temperature (RT) for 5 minutes to allow for complete disassociation of the nucleoprotein complex. 0.2ml of chloroform was subsequently added and the eppendorf vigorously shaken before being left to stand for 15 minutes at RT. The samples were then centrifuged (15,000g for 15min at 4°C) which generated three phases of sample; organic (protein), interphase (DNA) and aqueous phase (RNA). The RNA phase was aspirated, placed into a 1.5ml eppendorf, mixed with 1:10 isopropanol (Sigma) and left to stand at RT for 5 minutes following which the sample was centrifuged (12,000 x g, 10min, 4°C). The rest of the isopropanol (to give a final volume of 0.5ml) was then added and mixed, the sample stood at RT for 5 minutes and subsequently centrifuged (12,000 x g, 10min, 4°C). The centrifugation step left an RNA precipitate on the side of the tube, and from this point on the samples were kept on ice. The supernatant was removed and sample washed with 1ml of 70% ethanol, vortexed and centrifuged (12,000 x g for 5 minutes, 4°C). The supernatant was removed and the sample air dried for 5 minutes, following which the RNA pellets were dissolved in 50µl nuclease free water.

In order to determine the purity and concentration of the RNA, a small sample was taken and diluted 1:10 in nuclease free water. The sample was then analysed using A260/A280 spectrophotometry on a Birttek Powerwave XS microplate spectrophotometer (BioTek) and adjusted to 0.05mg/ml in nuclease free water.

2.4.2: cDNA synthesis

Using Taqman reverse transcription reagents (Applied Biosystems, UK), RNA samples (0.5µg in 10ml) were reverse transcribed. A master mix was made up consisting of 1x Taqman RT buffer (5µl), MgCl₂ (5.5mM, 11µL), deoxyNTP mix (500uM/NTP; 10µL), random hexamers (2.5µM; 2.5uL), RNase inhibitors (0.5U/µL; 1µL) and Multiscribe reverse transcriptase (1.25U/µL: 1.25µL) to give a final reaction volume of 50µL (All reagents from Applied Biosystems). The samples were then incubated for 10 minutes at 25°C, 30 minutes at 48°C and 2 minutes at 95°C in a thermal cycler (model 480; Perkin Elmer, Boston, USA). Samples were frozen at -80°C until required.

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2.4.3: Taqman real-time PCR

The cDNA produced was then used for RTPCR amplification using an ABI PRISM 7000 Taqman machine (Applied Biosystems). A reaction mix totalling 25µL was prepared containing 2x Taqman universal master mix containing Taq DNA polymerase (12.5µL), sample cDNA (10ng), Assay On Demand (AOD) reagent containing specific primers and probes (1.5µL) and 18s internal control (1.5µL) (All reagents from Applied Biosystems). The amplification protocol consisted of 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and finally the sample is kept for 1 minute at 60°C.

The Taqman RTPCR probes contain a fluorescent reporter dye at the 5' end and a quencher at the 3' end (e.g. FAM and TAMRA respectively). When the quencher is close to the reporter probe, fluorescence emission by the reporter dye is reduced, but as the Taq polymerase increases the primer, the 5' nuclease activity of the probe cleaves the probe annealed to the template strand releasing the fluorophore and separates the reporter from the quencher leading to an increase in fluorescence. Each new copy of cDNA transcribed causes an increase in fluorescence, and the fluorescence detected is directly proportional to the fluorophore released and amplicon produced.

The housekeeping gene 18s is used as a control, as this gene is present in all eukaryotic cells. This uses a different quencher dye (VIC) which can allow multiplex reactions where signals from target genes and control can be detected from the same reaction. The control signal can be compared to the target cDNA probe to give relative expression of the gene of interest.

2.4.4: Analysis

Samples were analysed using Sequence Detection Systems software (v 1.2.1, Applied Biosystems, UK) and the amount of target gene normalised to amount of 18s in the same cycle. A threshold value was set manually on the amplification plot on the geometric (linear) phase of amplification following guidelines supplied by Applied Biosystems. The Threshold Cycle (C_t) is the cycle number where fluorescence passes the threshold, and C_t values are inversely proportional to the initial copy number so a lower C_t value means a higher concentration of target in the sample. The gene expression data is expressed as arbitrary values using C_t values from reporter dyes of target normalised to the control 18s emission (ΔC_t). To take into account exponential amplification reactions the data was transformed and presented as $2^{-\Delta C_t}$. This allows the mRNA in different samples to be compared relative to expression of the endogenous control.

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2.5 Isolated jugular and nodose ganglia recordings

Once the gene of interest was found to be expressed at the mRNA level in the whole ganglia, a functional effect was then investigated using isolated jugular and nodose ganglia recordings. The effects of agonists on intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) from the ganglia were investigated as described in (Dubuis *et al.*, 2013). This technique allows the examination of airway terminating neurons using a retrograde lipophilic dye.

2.5.1: Labelling of airways neurons by retrograde tracking

12-14 days prior to the experiment, Male Dunkin Hartley guinea pigs were dosed with 1 ml/kg of 0.25 mg/ml DiI_{C₁₈} (3) (1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) (Invitrogen) intranasally (i.n), which ensured specific staining of airway innervating neurons by retrograde transport (Undem, 2002b). The DiI was dissolved in ethanol and diluted on the day of the experiment to give 2% V/V ethanol in 0.9% saline.

2.5.2: Ganglia dissection and dissociation

Guinea pigs were sacrificed by injection of pentobarbitone (200mg/i.p) and both nodose and jugular ganglia were carefully removed and placed in ice cold sterile Hanks Balanced Salt Solution (HBSS: containing 5.33M KCl, 0.441mM KH_2PO_4 , 138mM NaCl, 0.3mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.6mM glucose, 5mM HEPES and pH 7.4). The tissue was then cleared of connective tissue under the microscope and in sterile conditions and the nodose and jugular ganglia were enzymatically digested as described previously (Grace *et al.*, 2012; Dubuis *et al.*, 2013). Briefly, ganglia were digested in 2 steps: They were first incubated with activated papain (20 active units/ml, Sigma, UK) in Papain Activation Solution (PAS: HBSS supplemented with 400mg/L L-Cysteine, 1ml/L EDTA 0.5M stock solution and 1.5ml/L CaCl_2 1M stock solution) for 30 minutes at 37°C. The ganglia were then incubated for 40 minutes with Type IV collagenase (Worthington, USA, 2mg/ml) and Dispase II (Roche, UK, 2.4mg/ml) at 37°C in sterile HBSS. Once digested, the ganglia were washed by centrifugation, supernatant removal and the pellet resuspended in sterile HBSS. The cells were dissociated by trituration with a heat-smoothed glass Pasteur pipette coated with L15 medium. In order to separate the cells from unwanted cell types and remaining undigested tissue, the suspension was centrifuged through L15 medium containing 20% Percoll v/v and the neuron pellet was resuspended in L15 alone. After centrifugation the cells were resuspended in complete F12 medium (containing 1% FBS, 1% Penicillin and 100,000u/ml streptomycin) and adhered to Poly-D-Lysine and laminin (22.5µg/ml) coated fluorodishes. The cells were left to adhere for 2

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hours following which the plates were flooded with 2ml of with complete F12 medium and kept at 37°C (5%CO₂/95%O₂) for 16 hours prior to use. Fluorodishes containing cells were imaged within 24 hours.

2.5.3: Staining and imaging

The dishes containing the neurons were loaded using a ratiometric calcium sensitive dye (Fura2-AM, 3µM Invitrogen) for 40 minutes in the dark at 25°C, following which the dye was aspirated and replaced with ECS to allow for a de-esterification step for 30 minutes at 25°C. Fura2-AM has a different excitation and emission spectrum according to the binding state. If bound to calcium, Fura2 has a maximum excitation wavelength of $\lambda=340\text{nm}$ and emission wavelength of $\lambda=520\text{nm}$, whereas unbound has an excitation of $\lambda=380\text{nm}$ and emission at $\lambda=514\text{nm}$. Following de-esterification, the cells were washed with ECS (containing in mM: KCl, 5.4; NaCl, 136; MgCl₂, 1; CaCl₂, 1.8; NaH₂PO₄, 0.33; glucose, 10; HEPES 10 at pH 7.4 and 37°C) and the fluorodish mounted on a Widefield inverted microscope (Zeiss Axovert 200, Carl Zeiss Microscopy, UK). The microscope stage was surrounded by an incubation chamber maintained at 37°C and 5% CO₂, and contained a custom made perfusion system which allowed the delivery of compounds dissolved in ECS and a rapid changeover (3 seconds) between different compounds of interest while keeping the fluorodish at a constant volume of 600µL. All solutions were maintained at 37°C and 5% CO₂ throughout the whole experimental protocol. A Hamamatsu EM-CCD C9100-02 camera connected to simple PCI software was used to visualise the cells.

Neurons were identified using bright field illumination at x20 magnification. Airway specific cells could then be identified by imaging the cells at excitation and emission fluorescence wavelengths of $\lambda=520\text{ nm}$ and $\lambda=570\text{ nm}$, which identifies the Dil labelled cells. Intracellular Ca²⁺ ([Ca²⁺]_i) responses were then investigated as previously described (Grace *et al.*, 2012, Dubuis *et al.*, 2013). Neuron responsiveness and viability was assessed by application of K₅₀: a potassium chloride solution (containing in mM: 50mM KCl, 91.4mM NaCl, 1mM MgCl₂, 2.5mM CaCl₂, 0.3M NaH₂PO₄, 10mM glucose 1mM HEPES at pH 7.4) for 15 seconds at the start and end of recording, which also was used to normalise neuron responses. Following the K₅₀ response, the fluorodish was washed with ECS until the calcium signal returned to baseline. Agonist responses could then be carried out by perfusing the compound of choice onto the fluorodish for 10 minutes and following with a wash step with ECS. Throughout the experiment one image was taken of Fura2 emission following excitation at 340 nm and 380 nm in close succession every 10 seconds producing two parallel sets of images taken throughout the experimental protocol and used to

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produce the ratio of intensities. Only neurons producing a fast response to K_{50} which was easily washed, and that had a diameter of over 20 μ M were analysed.

2.5.4: Analysis

Each cell was individually analysed using Image J software (64-bit, version 1.43u, NIH, USA) and the ratio of the signal emitted at 520nm when excited at 340nm divided by the signal emitted at 520nm when excited at 380nm was calculated. This ratio accounts for any degradation of the dye by photo-bleaching and also inter-experimental differences in loading of Fura2-AM. The sequence of ratios was then uploaded into Origin software (Version 8.6, OriginLabs Corp, USA) to produce a graph of emission over time. The calcium signal induced by each agonist was calculated using total area under the curve, (A.U.C.) which reflects the change in intracellular calcium from resting level over time. This was then normalised to a percentage of the calcium signal (A.U.C.) induced by K_{50} application, so that differences between individual cell responses can be accounted for.

2.6 Isolated vagal nerve recordings

The isolated vagus preparation provides a high-throughput, pharmacological amenable technique, which allows the use of tissue from guinea pig and knock-out mice alongside donor human tissue. In addition, this *in vitro* model provided a preparation free from the complications of bronchoconstriction and mucus production that can occur *in vivo*, and may account for, or influence, the results seen.

2.6.1: Tissue Dissection: Animals

Male Dunkin Hartley guinea pigs (250-350g), WT (C57Bl/6j) or genetically modified mice (*Trpv1*^{-/-}, *Trpa1*^{-/-}, *Trpv4*^{-/-} and *Px1*^{-/-}) (18-20g) were euthanized via overdose of pentobarbitone i.p. (200mg/kg). The neck was opened by cervical incision to expose the trachea and thorax and tissue cleared until the vagal nerves were revealed. Following removal of the ribcage, segments of both vagus nerves caudal to the nodose ganglia were removed and placed into modified Krebs solution containing (in mM: NaCl 118; KCl 5.9; MgSO₄ 1.2; CaCl₂ 2.5; NaH₂PO₄ 1.2; NaHCO₃ 25.5 and glucose 5.6; pH 7.4) and bubbled with 95% O₂ / 5% CO₂. The nerve was cleared of any further connective tissue and (in the case of the guinea pig) carefully desheathed and cut into sections 10-15mm long prior to experimentation. Care was taken not to stretch or crush the nerve trunks and that they remained in oxygenated Krebs solution throughout the experiment.

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2.6.2: Tissue Dissection: Human

Whole human lungs unsuitable for transplant were purchased from IIAM, and received on ice within 36 hours of cross clamp. Upon arrival the lungs were placed immediately into Krebs solution and bubbled with 95% O₂ / 5% CO₂. Sections of the vagus nerve were identified running alongside the trachea and dissected free from surrounding and connective tissue. The perineural and epineural sheath is much harder to remove in human tissue, so care was taken to remove as much as possible under a dissecting microscope. Similarly to the guinea pig and mouse tissue, the nerves were then cut into sections 10-15mm long and kept in oxygenated Krebs throughout the experiment.

2.6.3: Vagal nerve recordings

Once dissected, the nerves were loaded into a grease-gap recording chamber (**Figure 2.1**) as previously described by (Birrell *et al.*, 2002; Maher *et al.*, 2009; Grace *et al.*, 2012). Each piece of nerve was pulled through a custom made Perspex chamber and each end of the nerve electrically and chemically isolated using petroleum jelly injected through a side arm. A glass rod was carefully filled to avoid any air bubbles with modified Krebs solution and placed into an Ag/AgCl pellet half-cell electrode (Mere 2 flexible electrodes, WPI, UK) and rested on one end of the nerve. A similar electrode was prepared and placed on the opposite end of the nerve. These were connected to a DAM50 differential amplifier unit (WPI, UK) which amplified the DC potential signal (x10 gain, high filter 1KHz, 5Hz sample rate) and fed back to a chart recorder (Lectromed 2, Lectromed, UK (now Digitimer, UK)). The chart recorder recorded depolarisation in mV and was calibrated so that 1mm was equal to 0.01mV. One end of the nerve (recording electrode) was constantly perfused with Krebs solution at a rate of approximately 2ml/minute, and the other end (reference electrode) was kept in a chamber containing Krebs solution (**Figure 2.1**). Krebs solution and agonists or antagonists dissolved in Krebs were kept in syringes above the chambers, oxygenated and maintained at 37°C using heated water jackets.

This technique measures the compound depolarisation caused by a difference in ionic potential of all nerve fibres within the nerve rather than single action potentials or specific nerve firing. When a drug which activates the nerve is perfused onto the end of the nerve fibre, this causes Na⁺ ions from the Krebs solution to move into the nerve cell, leaving excess Cl⁻ ions which interact with the AgCl pellet in the electrode to electrically stimulate the circuit. There would be no change in Cl⁻ ions in the reference electrode, causing a potential difference between the two ends of the nerve which is amplified and recorded as depolarisation on the chart recorder. When the

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stimulation is washed off with Krebs, K^+ ions leave the nerve cell which causes the Cl^- ions to disassociate from the AgCl pellet. The potential difference between the two ends of the nerve is lost and this is recorded as repolarisation.

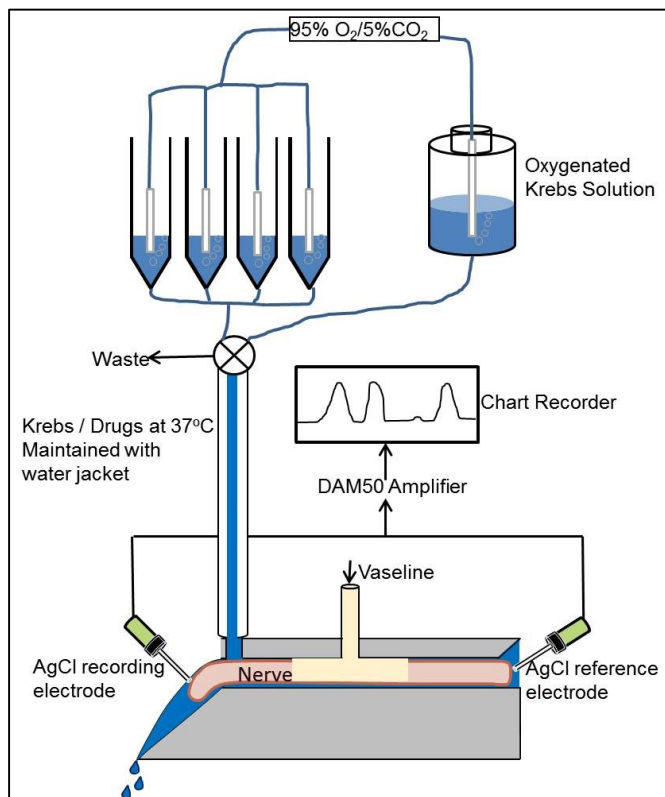


Figure 2.1: Isolated vagus set up

The nerve is drawn into a grease gap recording chamber where each end of the nerve is electrically and chemically isolated using Vaseline. One end of the nerve is constantly perfused with either Krebs solution or compound of interest at a rate of 2ml/min and connected to a recording AgCl electrode. All solutions are bubbled with 95%O₂/5%CO₂ and kept at 37°C by a water jacket connected to a water bath. The other end of the nerve is kept in Krebs for the duration of the experiment and connected to a reference AgCl electrode. When a stimulus is added to the recording end of the nerve, this causes a potential difference between the two ends of the nerve which is detected by the electrodes and amplified via a DAM50 differential amplifier and can be recorded as depolarisation on a chart recorder.

2.6.4: Agonists and antagonists experimental protocol

In order to determine if a compound caused activation of the nerve, non-cumulative concentration responses to potential tussive stimuli were carried out. This involved stimulating the nerve with single concentrations of an agonist for 2 minutes, following which the nerve was washed with

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Krebs until it returned to baseline, and this repeated with the full range of concentrations. A similar stimulation was also carried out with vehicle to ensure that it had no effect. Depolarisation was recorded in mV. Following characterisation, a sub maximal concentration of agonist was taken forward for antagonist experiments. A concentration response with a maximum of 5 stimulations could be carried out on each piece of nerve from any one animal or patient.

A classical pharmacological approach was used to investigate inhibition of agonist responses by antagonists. The nerve would be exposed to agonist for 2 minutes and then washed with Krebs until the response returned to baseline. This was repeated to provide two control agonist responses. The nerve would then be incubated with an antagonist for 10 minutes, and then re-stimulated with agonist in the presence of antagonist for 2 minutes 20 seconds (the extra 20 seconds is to allow for the changeover of stimuli) to assess if the antagonist was able to affect the magnitude of the depolarisation induced by the agonist. Following a brief washout period the nerve was exposed to agonist for 2 minutes to provide a recovery response to ensure nerve viability and that the antagonist was washed off (**Figure 2.2**). The inhibitory activity of the antagonist on the agonist responses was assessed by comparing the magnitude of depolarisation of the nerve following antagonist incubation to the mean magnitude of the two control agonist responses, to give a % inhibition. Only one concentration of antagonist was tested on each piece of nerve from any one animal or patient.

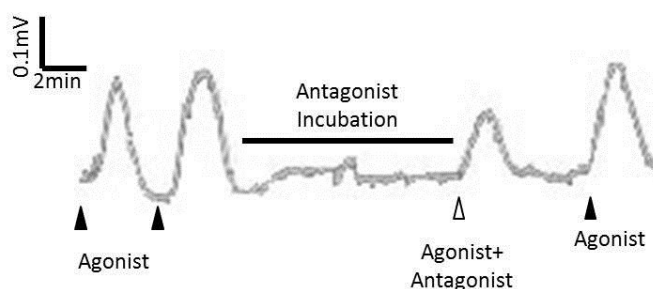


Figure 2.2: Example trace from an antagonist protocol

Once the nerve is loaded into the grease-gap recording chamber and a stable baseline obtained, two reproducible agonist responses are carried out following which the nerve is incubated with either antagonist or vehicle for a set period of time (normally 10 minutes). The nerve is then stimulated with agonist in the presence of antagonist, and response compared to the mean of the original two agonist responses. After a short washout period, the nerve is re-stimulated with agonist to ensure washout of antagonist and also viability of nerve tissue.

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Although high throughput and amenable to pharmacology, there are several limitations to consider with this technique. Responses are a summation of all events within the nerve trunk following treatment with an agonist and are not single action potentials as depolarisation induced by an agonist may not be of sufficient magnitude to generate an action potential. As drugs are applied to the nerve trunk, this may not be indicative of what happens *in vivo* at the nerve terminals as receptors may be present in a different confirmation, and also not all fibres within the trunk specifically innervate the airways. Nevertheless, depolarisation of the isolated vagus has been shown to be predictive of a cough response *in vivo* (Maher *et al.*, 2009), and results from both guinea pigs and human vagus have been shown to be comparable (Nasra and Belvisi, 2009). In addition, the key advantage of this technique is the use of human tissue to provide translational data. All results were repeated (where possible) using airway single fibres and *in vivo* cough.

2.7 *In vivo* single fibre experiments

An *in vivo* anaesthetised guinea pig single fibre preparation was used to investigate the effect of specific agonists on action potential firing of single afferent airway terminating fibres. This experimental technique involves anaesthetising a guinea pig and cutting both vagal nerves at the central end, using fibres from the left vagal nerve for recordings. Therefore any responses were effects on the nerve fibre itself and not as a result of a reflex arc. This had the advantage of measuring the effect of compounds on the nerve termini with an end point of nerve firing, and so was more physiologically relevant than both the isolated vagus and ganglia. In addition the effect of compounds on tracheal pressure as a measure of bronchoconstriction could be monitored in the absence of a reflex arc.

2.7.1: Surgery

Male Dunkin-Hartley guinea pigs (400-750g) were anaesthetised with urethane (1.5g kg^{-1}) i.p. with additional urethane supplied if required, and paralysed with vecuronium bromide (0.10mg/kg i.v. initially) and every 20 minutes thereafter (0.05mg/kg). Levels of anaesthesia were constantly monitored throughout. The guinea pigs were then prepared as described in (Adcock *et al.*, 2003), and summarised in **Figure 2.3**. Briefly, the trachea was cannulated and both blood gas and pH maintained at physiological levels using a ventilator (Ugo Basile small animal ventilator). The guinea pig was artificially ventilated at a tidal volume of 10ml/kg and 50-60 breaths per minute. A nebuliser (Aerogen nebuliser, Buxco) was used for the delivery of aerosolised compound of interest or vehicle. The nebulizer assembly was connected to the ventilator and arranged so that inspired air was passed through the medication chamber before entering the lungs of

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anaesthetized animals via the tracheal cannula. Both the right jugular artery and carotid were cannulated for the administration of drugs and to measure blood pressure respectively. Both blood pressure and heart rate were monitored throughout the experiment using a transducer (Gould P2X3L, MEMSCAP SP 844), and body temperature monitored using a rectal thermometer and maintained at 37°C using a heated blanket (Harvard Apparatus, UK). In addition to action potential firing, this technique also allows the measurement of tracheal pressure using an air pressure transducer (SenSymb647, MEMSCAP SP 844) attached to a side arm of the tracheal cannula.

Both cervical vagal nerves were then cut at the central end and dissected free from the carotid artery, sympathetic and aortic nerves. Only the left vagal nerve was used for recording, and this was cleared of all surrounding tissue and fascia, and the skin and muscle surrounding the incision tied to a metal ring to form a well and filled with light mineral oil. Recording of action potentials was undertaken using Bipolar Teflon coated platinum electrodes which were exposed at the tips. The reference electrode was placed on the nerve fascia. Using a binocular microscope, thin filaments of nerve were teased free from the whole vagus until a single active unit of no more than 2-3 fibres was obtained and placed on the recording electrode. These electrodes were connected to a pre amplifier headstage (Digitimer NL100k) and the signal from action potentials amplified (x1000-5000, Digitimer NL104), filtered (at a range of LF30Hz-HF8.5KHz, Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) before recording. All signals (including blood pressure, heart rate and tracheal pressure) were recorded using Spike 2 data acquisition software via a CED Micro 1401 interface. This software allowed pulse training counting over selected time period. The input signal was also monitored using a digital storage oscilloscope (Tektronix DPO 2012) and fed through an audio amplifier to a loudspeaker. Guinea pigs were euthanized by an overdose of pentobarbitone (i.p.) at the end of the experiment.

2.7.2: Nerve fibre identification

Each nerve fibre could be identified as originating from the family of SARs, RARs or chemosensitive C fibres using a set of criteria (Adcock *et al.*, 2003). These would include the pattern of spontaneous activity of the fibre, its response to hyper-inflation or deflation, Adaptation Induces (AIs), the response to capsaicin and citric acid, and conduction velocity. In brief if a fibre had little or no spontaneous activity, no response to hyperinflation or deflation, and responded to a capsaicin aerosol, it was assumed that the fibre was a C fibre. If the nerve fibre had a set pattern of spontaneous activity and responded to hyperinflation and deflation, and to citric acid, it was

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assumed to be an A δ fibre (RAR). Assumptions would be verified at the end of the experiment where conduction velocities would be measured.

Conduction velocities were measured using bipolar silver electrodes, which would stimulate the vagus nerve close to the thorax with a supra threshold of 0.5ms/1Hz (Grass stimulator). The action potential and stimulus would be recorded as a single sweep on Spike software and the time interval between them along with the distance between the stimulating electrode and recording electrode taken to calculate the conduction velocity.

2.7.3: Agonist experimental protocol

The following basic experimental protocol was carried out to investigate the effects of agonists on both C fibres and A δ fibres. Exact timings and agonists used are included in the relevant chapter.

Once a lung afferent fibre had been identified, a baseline recording was taken for 2 minutes. Vehicle of the compound of interest was administered into the lungs for a 1 minute period during which any changes in fibre activity, intratracheal pressure and blood pressure were continuously recorded until they returned to baseline levels. After an interval of 10-20 minute, capsaicin (100 μ M) was administered for 20 seconds and changes in fibre activity, intratracheal pressure and blood pressure were continuously recorded until they returned to baseline levels. 10-20 minutes following this, citric acid (0.3M) was administered for 1 minute during which changes in fibre activity, intratracheal pressure and blood pressure were continuously recorded until they returned to baseline levels. The compound of interest was then administered for up to 1 minute (by aerosol) and changes in fibre activity, intratracheal pressure and blood pressure were continuously recorded until they returned to baseline levels. If experimentation was carried out on a C fibre, a further capsaicin aerosol was given, and if an A δ fibre used then a further citric acid aerosol was used following exposure to the agonist to ensure that the fibre was still responsive.

Data was expressed as mean $\pm$ SEM and analysed using paired students t-test comparing responses as absolute values after stimulus to baseline values immediately before the response.

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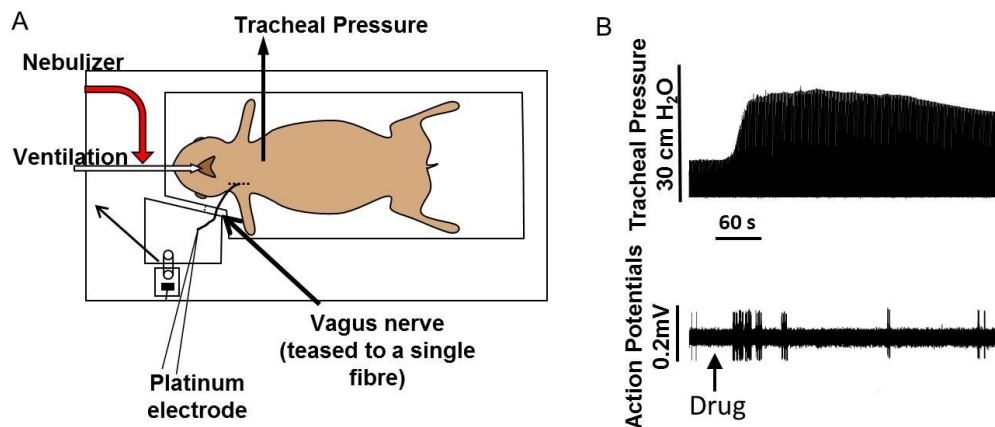


Figure 2.3: *In vivo* single fibre recording set up and example trace

Guinea pigs were anaesthetised and ventilated and both vagal nerves cut. The left vagal nerve teased to a single fibre and attached to a platinum electrode as shown in (A). Compounds of interest could then be aerosolised and administered directly to the lungs using a nebuliser. Changes in trachea pressure and fibre firing could then be measured and recorded, and an example trace is shown in (B). The top panel represents an increase in tracheal pressure following administration of a test compound, and the bottom trace is an example of an increase in fibre firing following compound administration.

2.8 *In vivo* guinea pig cough recordings

This technique was used to assess the tussive capability of a compound of interest in a conscious, naïve guinea pig. Unlike rats and mice, guinea pigs have a cough response similar to humans (Maher *et al.*, 2009) and therefore if a compound causes cough in the guinea pig, it can be assumed that it could also be a tussive stimulus in humans.

Conscious, unrestrained guinea pigs (250-300g) were placed in individual transparent Perspex plethysmography chambers (Buxco, Wilmington NC, USA) (**Figure 2.4**). The chambers were attached to a central Aerogen nebuliser (Buxco, US), which allowed simultaneous aerosolisation of a tussive stimuli and visualisation of the responses of 2 guinea pigs at one time. Both chambers were equipped with a microphone connected to an amplifier and a speaker. Cough was detected as previously described (Birrell *et al.*, 2009; Maher *et al.*, 2009). Briefly, a potential tussive stimulus would be aerosolised for 5 minutes, and coughs manually counted by two trained observers which were blinded to treatment for 10 minutes. The mean of the counts by the two observers was used. Coughs were recognised by the characteristic stance and posture of the animal, the sound produced and also the change in flow recording (**Figure 2.4**). For antagonist studies, guinea pigs would be dosed with antagonist (i.p.) at a set time period prior to aerosol

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stimulation with agonist. Guinea pigs would be monitored for any adverse health effects during this time period. At the end of the experiment, guinea pigs would be euthanised with an overdose of pentobarbitone (200mg/kg i.p.).

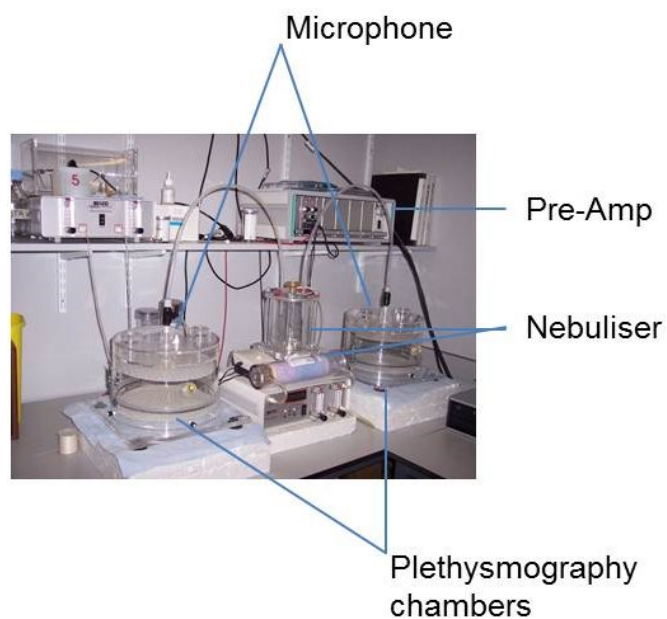


Figure 2.4: Cough chamber set up

Animals were placed into individual plethysmography chambers equipped with microphones (as shown in A), and a potential tussive stimulus was nebulised directly into each chamber. The number of coughs to a given stimulus were then counted by two trained observers blinded to treatment.

Chapter 3:

Investigating a role for TRPV4 in airway sensory nerves

3. Investigating a role for TRPV4 in airway sensory nerves

3.1: Rationale

A number of members of the family of TRP ion channels have been shown to modulate a variety of functions in the airways including inflammation, airway smooth muscle tone and activation of airway afferents (Bessac and Jordt, 2008; Nassini *et al.*, 2010). Two members of this family; TRPV1 and TRPA1 are well documented to play a role in airway sensory nerve activation and cough (Andrè *et al.*, 2009; Birrell *et al.*, 2009; Grace *et al.*, 2012; Lieu *et al.*, 2012), however, the role of other TRP channels such as TRPV4 in the lung, and particularly in modulation of airway sensory nerve activity, is relatively unexplored.

TRPV4 is a calcium permeable polymodal receptor expressed in human airway smooth muscle cells, macrophages, pulmonary arterial smooth muscle and epithelial cells (Jia *et al.*, 2004; Alvarez *et al.*, 2006; Dietrich *et al.*, 2006; Yang, 2006; Fernández-Fernández *et al.*, 2008, Baxter *et al.*, 2014; Hamanaka *et al.*, 2010). TRPV4 expression has also been found in DRG neurons (Grant *et al.*, 2007) and also at the mRNA level in pulmonary sensory neurons (Ni *et al.*, 2006). However a role in airway sensory nerves is yet to be established. Many endogenous activators of TRPV4, including arachidonic acid derivatives (Watanabe *et al.*, 2003) and an altered airway osmolarity (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Jia *et al.*, 2004) are released or altered in the airways of patients with asthma and COPD (Naruyima *et al.*, 1999, Joris *et al.*, 1993, Rolin *et al.*, 2005), where the cough response and therefore airway sensory nerve responses are altered. Therefore it could be hypothesised that TRPV4 could play a role in airway sensory nerve activation and cough.

3.1.1: Chapter Hypothesis:

Activation of TRPV4 on airway sensory nerves by selective agonists leads to nerve activation and cough

3.1.2: Aims:

- To evaluate the effect of two synthetic TRPV4 agonists, the phorbol derivative 4-alpha-phorbol-12,13-didecanoate (4αPDD, (Watanabe *et al.*, 2002), and GSK1016790a (GSK101) (Thorneloe *et al.*, 2008), in airway sensory nerves *in vitro*. The tools will be assessed using calcium imaging of guinea pig airway specific ganglia and vagal nerve depolarisation in isolated murine, guinea pig and donor human tissue.

3. Investigating a role for TRPV4 in airway sensory nerves

- The more potent of the two ligands, GSK101 will then be taken forward *in vivo*, initially to test whether it is able to propagate an all or nothing action potential in guinea pig isolated single fibres.
- Finally GSK101 will then be investigated in the whole animal where its ability to cause cough in the conscious guinea pig will be evaluated.

3. Investigating a role for TRPV4 in airway sensory nerves

3.2: Methods

3.2.1: RT-PCR

Quantitative real time PCR assays for the TRPV4 receptor were purchased from Applied Biosystems and validated using cDNA from tissue/organs from the guinea pig that expressed the target channel at high levels.

Whole nodose and jugular ganglia were harvested from male Dunkin Hartley guinea pigs. Expression levels of TRPV4 was determined using the validated assays, with real time RT-PCR performed as described previously (Wong, Belvisi, and Birrell, 2009) and in [Section 2.4](#).

3.2.2: Ganglia imaging

Male Dunkin Hartley guinea pigs were intranasally dosed with the retrograde tracer dye Dil to identify airway specific neurons, 14 days prior to dissection and enzymatic digestion of the nodose and jugular ganglia as described previously in [Section 2.5](#). Neurons were loaded into fluorodishes coated with laminin and allowed to adhere for 12 hours at 37°C 5%CO₂/95% O₂.

Following incubation, cells were loaded with the calcium specific dye Fura-2-AM for 40 minutes at 25°C in the dark, which was followed by a de-esterification step for 30 minutes at 25°C in the dark. Following a wash step with ECS, each fluorodish containing neurons was then placed in a full incubation chamber and mounted on a Widefield inverted microscope as described in [Section 2.5](#). Neurons were then identified using bright field illumination at x20 magnification, and airway specific cells could then be identified by imaging the cells at excitation and emission fluorescence wavelengths of approximately $\lambda=520-550\text{nm}$ and $\lambda=570$, which identifies the Dil labelled cells. Intracellular Ca²⁺ ([Ca²⁺]_i) responses were then investigated as previously described (Grace *et al.*, 2012; Dubuis *et al.*, 2013).

3.2.2.1: Agonist Concentration Response Curves

In order to determine if activation of TRPV4 was able to cause a change in [Ca²⁺]_i signal in the nodose and jugular ganglia, the following experimental protocol was undertaken. Throughout the experiment, neurons were constantly superfused with ECS using an in house pressurised perfusion system that allows complete change of solution (600µL) in 3s. The neurons were exposed to a 50mM potassium solution (K₅₀) for 10s to ensure neuron viability, and allowed to return to baseline. One concentration of the selective TRPV4 agonist (GSK101; 10-300nM) was then added to the dish for 10 minutes, and changes in [Ca²⁺]_i recorded.

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3.2.2.1: Analysis

Only responding cells (cells with a response $\geq 10\%$ of the K_{50} response) and cells with a diameter of over 20 μ m were analysed as these were more likely to be sensory neurons (Hunter *et al.*, 2000). Intracellular calcium signal induced by each agonist was calculated using total area under the curve, (A.U.C.) which is the total change in intracellular calcium from resting level over time. This was then normalised to a percentage of the total change induced by K_{50} application, so that differences between individual cell responses can be accounted for. The data is presented as mean $\pm$ S.E.M., N indicates number of animals, and n indicates number of cells.

3.2.3: Isolated vagal nerve recordings

Vagal tissue was dissected from male Dunkin-Hartley guinea pigs, WT C57/Bl6 mice and *Trpv4*^{-/-} mice and placed in a grease-gap recording chamber as previously described in [Section 2.6](#). Key experiments were repeated using human donor tissue to provide translational data.

3.2.3.1: Agonist concentration response curves

In order to determine if activation of TRPV4 was able to depolarise the vagal nerve, the following experimental protocol was undertaken. Non-cumulative concentrations of the TRPV4 agonists (GSK101; 0.03-1 μ M and 4 α PDD; 0.3-10 μ M) were applied to the vagal nerve trunk followed by a wash period where the nerve was washed with Krebs solution until it returned to baseline. In addition, vehicle (0.1% DMSO v/v) was tested on the nerve to ensure that it had no effect. Responses were recorded as mV depolarisation. Concentration ranges of each agonist were determined from published pEC50 values. GSK101 has previously been determined to have a pEC50 value ranging from 8.0 in rat to 8.7 in human tissue (Thorneloe 2011), and 4 α PDD is known to be less potent, with a published pEC50 value of 6.9 in mouse (Xu, Satoh, and Iijima 2003). Responses were carried out in guinea pig and WT mouse tissue, and a submaximal concentration of each agonist used to determine activation of human tissue.

3.2.3.2: Antagonist concentration response curves

Depolarisation by each agonist was then tested against specific TRPV4 antagonists. There are several known TRPV4 antagonists, of which two have been used in this thesis, the selective TRPV4 inhibitor HC067047, which has been shown to block TRPV4 activation in a bladder model in mice and rats (Everaerts *et al.*, 2010) and GSK2193874, an orally active antagonist shown to

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block TRPV4 activity in a model of heart failure induced pulmonary oedema (Thorneloe *et al.*, 2012). Both are thought to be highly selective for the TRPV4 ion channel.

A submaximal concentration of both GSK101 (300nM) and 4αPDD (1μM) were taken forward for inhibitor studies. Concentration-responses for the TRPV4 selective inhibitors HC067047 (0.1-10μM), GSK2193874 (0.1-10μM) (or vehicle (0.1% dimethyl sulfoxide (DMSO) v/v in Krebs) were established against both GSK101 and 4αPDD on guinea pig and wild type mouse vagus. A classical pharmacological approach was used; two reproducible responses to each agonist was established, following which each nerve was incubated with a single concentration of one of the antagonists for ten minutes. Following incubation, the nerve would be restimulated with agonist in the presence of antagonist to determine inhibition. The nerve would then be washed with Krebs until returned to baseline, and a final response to the agonist carried out to ensure nerve viability. From this, the concentration of antagonists exhibiting maximal inhibition of its receptor (10μM) was chosen for inhibition of activation of human tissue and for further experiments.

3.2.3.3: Determining specificity of tools

The selectivity of the available pharmacological tools was then established. Firstly the two TRPV4 agonists were tested on *Trpv4*^{-/-} vagal tissue. Knockout of the *Trpv4* gene was confirmed using standard genotyping techniques as described in [Section 2.3](#). Responses to both GSK101 and 4αPDD were established in both *Trpv4*^{-/-} tissue, and in age matched wild type controls. In order to ensure responses to other TRP agonists were not altered in the *Trpv4*^{-/-} tissue, responses to capsaicin (1μM; TRPV1 agonist) and acrolein (300μM; TRPA1 agonist) were also established in knockout and wild type vagus.

To determine agonist specificity, GSK101 and 4αPDD induced depolarisation was tested against TRPA1-selective (10 μM; HC-030031) and TRPV1-selective (100 μM JNJ17203212) antagonists on both guinea pig and mouse vagus. Furthermore, to ensure that the antagonist was not exhibiting off target effects on TRPV1 and TRPA1 receptors, HC067047 (10 μM) and GSK2193874 (10μM) were tested against acrolein (300 μM) and capsaicin (1 μM) induced vagal nerve depolarisation. Vehicle for all antagonists was 0.1% DMSO v/v in Krebs. Concentrations for the TRPA1 and TRPV1 antagonists had been previously established (Grace *et al.*, 2012).

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3.2.3.4: Analysis

Individual nerve depolarisations were measured in mV, and the chart recorder calibrated so that 1mm was equal to 0.01mV. Antagonist inhibitions were calculated by comparing the magnitude of depolarisation of the nerve following antagonist incubation to the mean magnitude of the two control agonist responses, to give a % inhibition. Statistical analysis for agonist concentration responses was carried out using a Kruskal Wallis test with a Dunns post-test comparing responses to vehicle. For antagonist studies; a two tailed t-test for matched pairs was used comparing the magnitude of depolarisation by the agonist with and without the presence of antagonist in the same piece of nerve. The data is presented as mean $\pm$ S.E.M., with statistical significance set at $P < 0.05$.

3.2.4: Genotyping of *Trpv4*^{-/-} Mice

Homozygous breeding pairs of mice genetically modified to disrupt the TRPV4 gene (*Trpv4*^{-/-}; RBRC No.01939) were obtained from the Riken Bioresource centre (Tsukuba, Japan) (Mizuno *et al.*, 2003; Suzuki *et al.*, 2003) and breeding colonies maintained on a C57Bl/6 background at Imperial College London. Regular genotyping was carried out to ensure continued deletion of the TRPV4 allele. This involved amplification of the DNA sequence of interest using PCR as previously described in [Section 2.3.1](#).

DNA samples extracted from the tail tips of WT (C57Bl/6) and *Trpv4*^{-/-} mice were amplified by PCR using primers (Invitrogen) and visualised on an agarose gel by electrophoresis. Samples were heated to 94°C, following which 40 cycles of denaturing, annealing and extension were carried out, the exact duration and temperatures of which are outlined in **Table 3.1**. The resulting PCR products were run on 2% agarose gel (80V for 1 hour) in TBE buffer containing 0.05 μ l/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) alongside a 50-2000bp ladder (Hyperladder 50bp, Bioline Reagents, UK). The expected wild type product was 740bp and knock out 1490bp.

Table 3.1: PCR Protocol for *Trpv4*^{-/-} mice

	PCR Conditions			
TRPV4	Denaturing 95°C 30 sec	Annealing 60°C 30 sec	Extension 72°C 1min20sec	Product Size (Base Pairs) WT: 740 KO: 1490

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3.2.5: *In vivo* single fibre recordings

Male Dunkin Hartley guinea pigs (~350-400g) were anaesthetised with urethane (1.5g/kg i.p.) and paralysed with vecuronium bromide (0.1mg/kg i.v.) and ventilated. Heart rate, blood pressure and body temperature were constantly monitored for the duration of the experiment. Guinea pigs were then prepared as described in [Section 2.7.1](#) and as previously described (Adcock *et al.*, 2003). Briefly, both vagal nerves were cut at the cervical end but only the left vagal nerve used for recording. This 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. The nerve fibre was identified as originating from the family of SARs, RARs or chemosensitive C fibres using a set of criteria (Adcock *et al.*, 2003) and as described in [Section 2.7.3](#). This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, Adaptation Indices, response to capsaicin and citric acid and finally conduction velocity which was determined at the end of the experiment.

3.2.5.1: *Agonist responses*

The following experimental protocol was undertaken to determine the effect of GSK101 on airway terminating vagal fibres. Following surgery, the animals were left to stabilise for 30 minutes, following which a control baseline recording was taken for 2 minutes. Vehicle (0.1% DMSO in 0.9% saline) was then aerosolised into the airways for up to 1 minute, and changes in fibre activity, intra-tracheal pressure and blood pressure were constantly monitored until they returned to baseline or a steady state achieved. 20 minutes later, citric acid (0.3M) was administered via aerosol into the airways and responses monitored. A further 20 minutes following return to baseline, GSK101 (10µg/kg; a concentration 10 fold higher than that tested *in vitro*) was aerosolised into the airway and changes in fibre activity, intra-tracheal pressure and blood pressure closely monitored. If a C fibre was under investigation, aerosolised capsaicin (100µM) would be administered to the airways at least 30 minutes following the response to the TRPV4 agonist.

3.2.5.2: *Antagonist responses*

For antagonist studies only Aδ fibres were investigated, and a lower concentration of GSK101 (100ng/ml) was used to allow for responses to return to baseline. Two reproducible responses to GSK101 (100ng/ml) were carried out 20 minutes apart, following which either the TRPV4 antagonist GSK2193874 (300mg/kg) or vehicle (6% Cavitron 2Hydroxypropyl-β-cyclodextrin in

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saline) was administered i.p. One hour later, GSK101 (100ng/ml) was aerosolised into the airways and changes in fibre activity, intra-tracheal pressure and blood pressure closely monitored.

3.2.5.3: Analysis

The data is expressed as mean $\pm$ S.E.M. and agonist studies analysed using paired students t-test comparing responses as absolute values after stimulus to baseline values immediately before the response. For antagonist responses in the same fibres a paired t-test was used comparing agonist responses following antagonist to control responses. For responses in different fibres, an unpaired t-test was used comparing responses in antagonist fibres to vehicle fibres.

3.2.6: Cough

Conscious, unrestrained guinea pigs were placed in individual transparent Perspex plethysmography chambers (Buxco, Wilmington NC, USA), as described in [Section 2.8](#). Cough was detected as previously described (Maher *et al.*, 2009; Birrell *et al.*, 2009.). An initial concentration response to GSK101 (1-30 μ g/ml), or vehicle (1% ethanol, 1% TWEEN in saline v/v) was carried out in each guinea pig. GSK101 was aerosolised for 5 minutes, and coughs counted for 10 minutes using two trained cough observers blinded to treatment. Coughs were recognised by the stance of the animal, the characteristic cough sound and changes in airflow. Once a submaximal concentration of GSK101 had been determined, the cough response elicited by GSK101 was tested against the specific antagonists HC067047 (100mg/kg) or GSK2193874 (300mg/kg). Guinea pigs were injected i.p. with a single antagonist or vehicle (1% DMSO in 0.9% saline v/v for HC067047, or 6% Cavitron/2-Hydroxypropyl- β -cyclodextrin in saline v/v for GSK2193874), 1 hour prior to exposure to GSK101 (3 μ g/ml). GSK101 was aerosolised for 5 minutes, and the number of coughs counted for 10 minutes.

3.2.6.1: Analysis

In order to test inhibition of cough by the TRPV4 antagonists, a Mann Whitney U test was used, which compared responses from the antagonist group to vehicle control. The data is presented as median $\pm$ interquartile range, with statistical significance set at $P < 0.05$.

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3.3: Results

3.3.1: TRPV4 expression in nodose and jugular ganglia

The nodose and jugular ganglia are known to hold the cell bodies for airway terminating vagal fibres. Prior to analysis of the ganglia, it was first confirmed that TRPV4 was expressed in guinea pig tissue, as guinea pigs are the only rodent known to have a cough reflex similar to that of man. RTPCR was carried out as described in [Section 2.4.3](#) in a variety of tissues, and RNA from the tissue with the most abundant expression, in this case the kidney, was serially diluted and used for validation (**Figure 3.1A**). A further PCR experiment was carried out with the diluted tissues and graph was plotted of log cDNA vs Ct. The assay was found to be valid as the lines corresponding to TRPV4 and the internal control (18s) were parallel straight lines with difference in slopes < 0.1 and R_2 value > 0.98 , therefore showing both assays to be equally efficient (**Figure 3.1B**). Following validation, quantitative RTPCR of whole nodose and jugular ganglia was carried out to investigate levels of TRPV4 mRNA. TRPV4 was determined to be expressed at the mRNA level in both ganglia, in accordance to previously published data (Ni et al. 2006) (**Figure 3.1C**).

3. Investigating a role for TRPV4 in airway sensory nerves

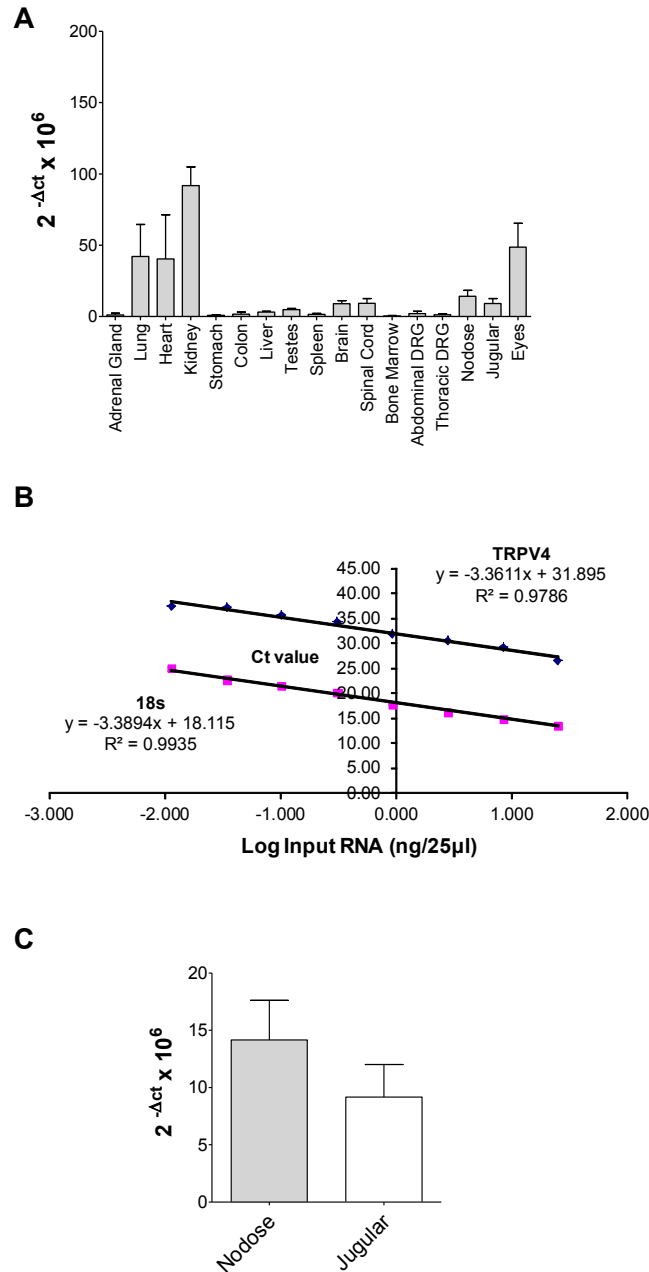


Figure 3.1: mRNA expression of TRPV4 in whole ganglia

(A): RTPCR was carried out on a variety of tissues from the guinea pig, where the kidney was found to have the most abundant expression. (B) The assay was found to be valid as the lines corresponding to TRPV4 and the internal control (18s) were parallel straight lines with difference in slopes <0.1 and R_2 value > 0.98 , therefore showing both assays to be equally efficient. (C) TRPV4 is expressed at the mRNA level in both nodose and jugular ganglia, with a higher level being present in the nodose. The data is expressed as mean $\pm$ S.E.M., $n=4$, and is normalised to the 18s control.

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3.3.2: Effect of TRPV4 on $[Ca^{2+}]_i$ in nodose and jugular ganglia

Once TRPV4 was known to be expressed at the mRNA level in whole ganglia, a functional effect was investigated using $[Ca^{2+}]_i$ signal in guinea pig isolated nodose and jugular ganglia neurons, where a change in $[Ca^{2+}]_i$ response is indicative of activation. Following a full concentration response to the TRPV4 agonist, GSK101 was shown to cause a concentration-dependent increase in $[Ca^{2+}]_i$ signal in airway specific nodose, but had less effect in the jugular ganglia neurons (**Figure 3.2 D-E**). Maximum $[Ca^{2+}]_i$ signals in responsive cells from the nodose ganglia were $132\% \pm 50\%$ of the K_{50} A.U.C. at 300nM of GSK101 (**Figure 3.2 A-C**). There were a total of 36 airway nodose cells examined, of which 75% responded to GSK101, and 34 jugular airway cells of which only 12% were responsive to GSK101 stimulation (**Figure 3.2D**). Non-airway neurons indicated a similar pattern wherefrom a total of 58 non-airway nodose neuronal cells, 52% were responsive, and of the 46 non-airway jugular neurons, 20% responded to GSK101 stimulation.

3. Investigating a role for TRPV4 in airway sensory nerves

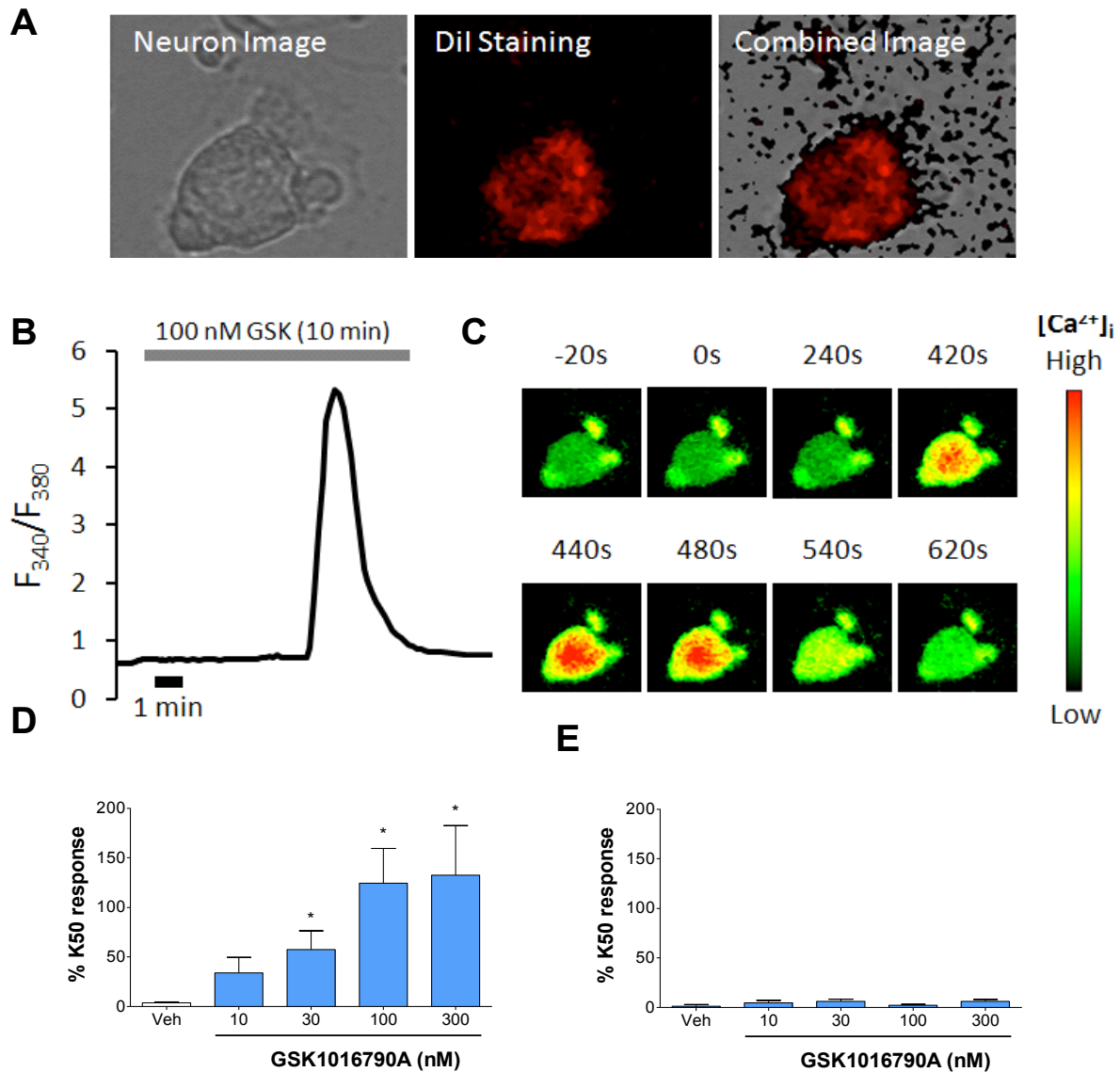


Figure 3.2: Effect of TRPV4 on $[Ca^{2+}]_i$ in nodose and jugular ganglia

A-C indicate example images and traces from $[Ca^{2+}]_i$ induced by GSK101 in nodose cells. A: Bright field and Dil stained cell indicating that the neuron is airway specific. B: An example trace of $[Ca^{2+}]_i$ induced by GSK101 in a nodose cell, shown as changes in light intensity over time. The grey bar indicates administration of agonist and incubation time. C: Real time images of calcium release induced by 100nM GSK101. The numbers above each panel indicate number of seconds, with 0seconds indicating GSK101 administration. Increase in red indicates increase in $[Ca^{2+}]_i$ with an colour indicator to the right. D: GSK101 caused a concentration-dependent increase in $[Ca^{2+}]_i$ as shown by % K₅₀ AUC, with a peak response at 300nM. E: However, there was no effect of GSK101 on jugular neurons.

The data is presented as mean $\pm$ S.E.M. of N=4-6, n=4-11. * indicates statistical significance ($p < 0.05$), Kruskal Wallis test with Dunns post-test comparing responses to vehicle.

3. Investigating a role for TRPV4 in airway sensory nerves

3.3.3: Effect of TRPV4 agonists on depolarisation of isolated vagal nerves

3.3.3.1: Effect of selective TRPV4 agonists

The *in vitro* isolated vagal nerve technique provides an airway relevant model free from problems such as bronchoconstriction or mucus production that can occur *in vivo*, where a functional role for TRPV4 in sensory nerves can be demonstrated. A further advantage is that translational data can be obtained using donor human tissue. The ability of two synthetic ligands GSK101 and 4αPDD to cause depolarisation of both guinea pig, murine and human isolated vagal nerves was investigated.

The TRPV4 specific ligands GSK101 and 4αPDD caused a concentration dependent depolarisation of both guinea pig (**Figure 3.3 A,B**) and mouse (**Figure 3.3 C,D**) vagal tissue. GSK101 (1μM) caused a maximum depolarisation of $0.21\text{mV} \pm 0.024\text{mV}$, and 4αPDD (3μM) caused a maximum depolarisation of $0.21 \pm 0.038\text{mV}$ in guinea pig tissue, which were both around 50-70% of the depolarisation induced by capsaicin (1μM). In mouse tissue, GSK101 (1μM) caused a maximum depolarisation of $0.25\text{mV} \pm 0.039\text{mV}$, and 4αPDD (3μM) $0.20\text{mV} \pm 0.028\text{mV}$, which was again around 50-70% of the control capsaicin response.

Sub maximal concentrations of GSK101 (300nM) and 4αPDD (1μM), chosen from the concentration responses in guinea pig and murine tissue, were also able to activate isolated human vagus nerve (**Figure 3.3 E**). GSK101 was able to cause a depolarisation of $0.10\text{mV} \pm 0.039\text{mV}$ and 4αPDD $0.087\text{mV} \pm 0.01\text{mV}$ in the human vagus.

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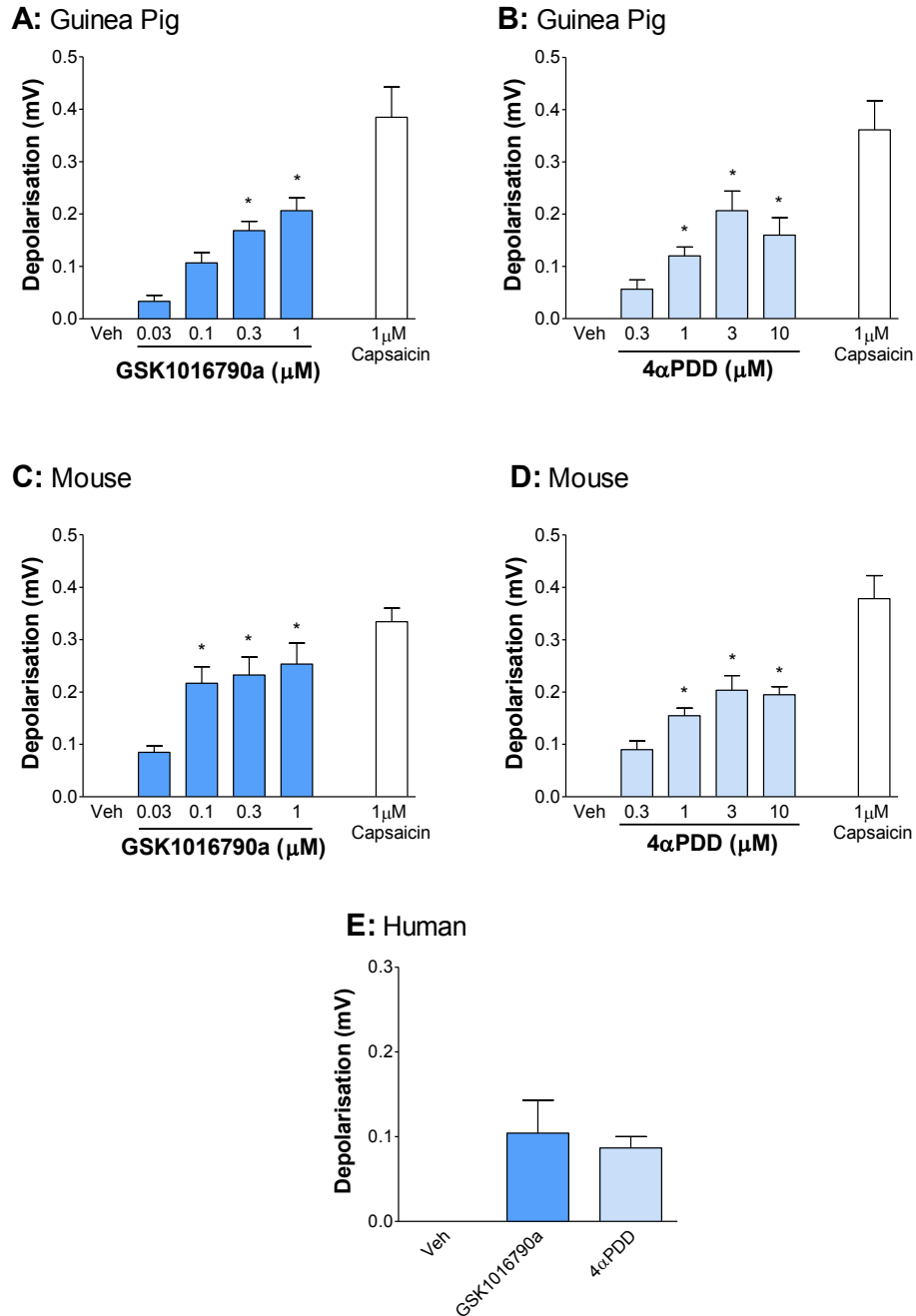


Figure 3.3: Effect of TRPV4 agonists on activation of isolated vagal nerves

GSK101 and 4αPDD caused concentration-dependent depolarisation of guinea pig (A,B) and murine (C, D) tissue. Submaximal concentrations of both GSK101 (300nM) and 4αPDD (1μM) were also able to cause depolarisation of human vagal tissue. The data is shown as mean ± S.E.M. of n=4-6 for guinea pig and mouse tissue, and n=3 for human and human tissue was obtained from 3 male donors aged 47-57 with no known respiratory disease. * indicates statistical significance at p<0.05 using Kruskal Wallis test and Dunns post-test comparing responses to vehicle.

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3.3.3.2: *Effect of selective TRPV4 antagonists*

The ability of two structurally distinct TRPV4 antagonists, HC067047 and GSK2193874 to inhibit TRPV4 dependent depolarisation was then investigated. A concentration response (0.1-10 μ M) to each antagonist against GSK101 (300nM) was initially carried out (**Figure 3.4**). HC067047 (10 μ M) was able to inhibit GSK101 induced depolarisation in both guinea pig and mouse tissue (**Figure 3.4 A,D**). In guinea pigs, depolarisation was inhibited 86% $\pm$ 9% in the presence of the maximally effective concentration of HC067047 (10 μ M) and in mice, depolarisation was inhibited 93% $\pm$ 4% by the same concentration of antagonist. HC067047 (10 μ M) also inhibited 4 α PDD (1 μ M) induced depolarisation to 95% $\pm$ 4% in guinea pig and 86% $\pm$ 8% in mouse tissue (**Figure 3.4 B,E**).

The alternate TRPV4 antagonist GSK2193874, showed a similar profile against GSK101 in the guinea pig. At the maximal effective concentration (10 μ M), it was able to inhibit GSK101 (300nM) induced depolarisation 93% $\pm$ 7% (**Figure 3.4 C**).

The maximal effective concentrations of HC067047 (10 μ M) and GSK2193874 (10 μ M) were investigated against GSK101 (300nM) and 4 α PDD (1 μ M) induced depolarisation in human donor tissue. Both antagonists were also able to inhibit TRPV4 induced depolarisation to around 80-100%, similar to what was seen in rodent tissue (**Figure 3.5 A, B**).

3. Investigating a role for TRPV4 in airway sensory nerves

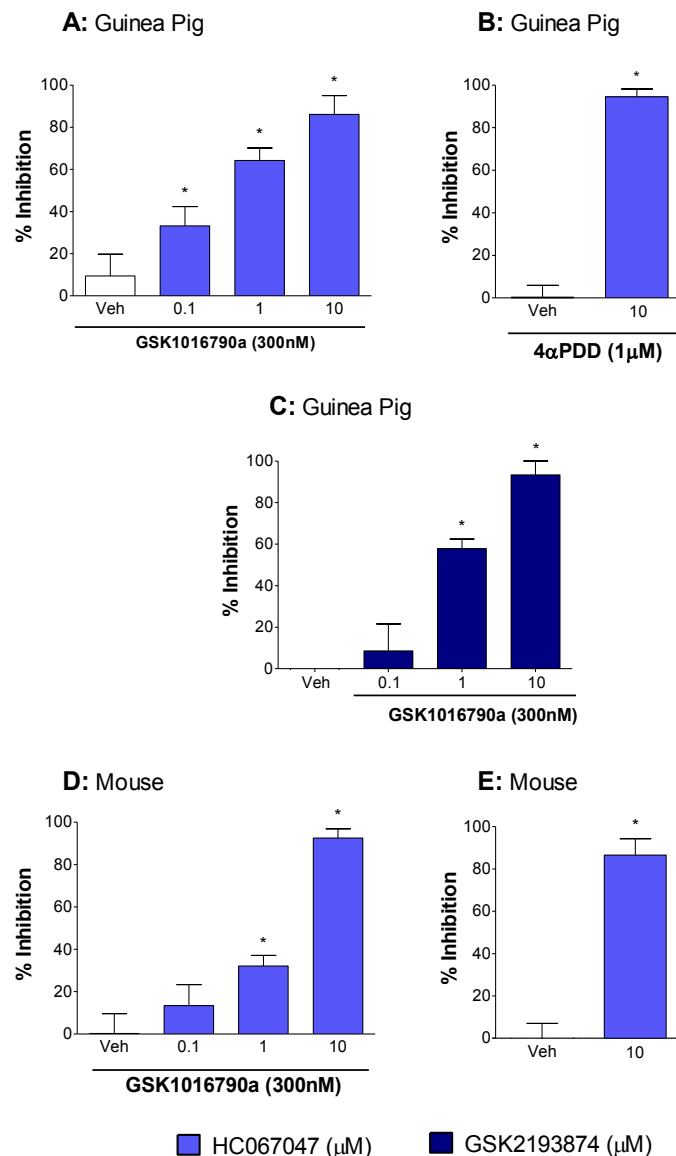


Figure 3.4: Effect of selective TRPV4 antagonists on TRPV4 induced depolarisation

The TRPV4 specific antagonist HC067047 inhibited GSK101 (300nM) induced depolarisation in a concentration dependent manner in (A) guinea pig and (D) mouse tissue, with a maximal effect at 10μM. This maximal concentration was able to inhibit depolarisation induced by 4αPDD to around 90% in both guinea pig (B) and mouse (E) tissue. (C): An alternate TRPV4 antagonist GSK2193874, also caused a concentration dependent inhibition of depolarisation induced by GSK101 (300nM) with similar potency to HC067047 in the guinea pig.

Vehicle (Veh; 0.1% DMSO) had no effect on nerve activation induced by either agonist in any species tested. The data is presented as mean ± S.E.M. of n=4-6 observations. * indicates statistical significance ($p < 0.05$), paired t-test comparing responses in the same piece of nerve with and without antagonist.

3. Investigating a role for TRPV4 in airway sensory nerves

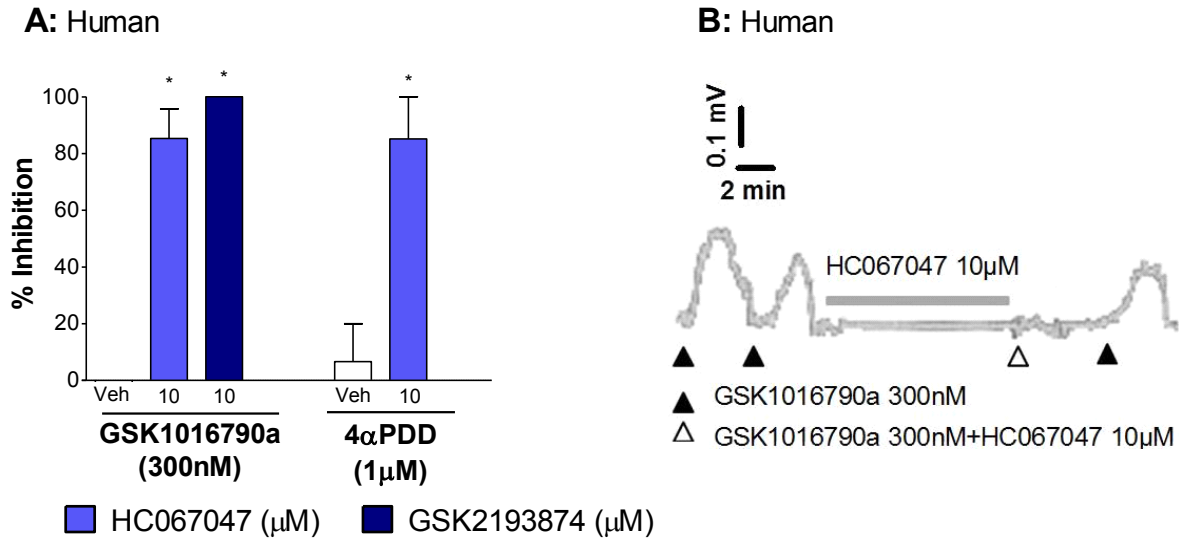


Figure 3.5: Effect of selective TRPV4 antagonists on TRPV4 induced depolarisation in human tissue

(A) Both antagonists were also able to inhibit depolarisation in human tissue, with an example trace indicated in (B).

Vehicle (Veh; 0.1% DMSO) had no effect on nerve activation induced by either agonist in any species tested. The data is presented as mean $\pm$ S.E.M. $n=2-3$ observations, and human tissue was obtained from 2 female and 1 male donor aged 38-70 with no known respiratory disease. * indicates statistical significance ($p < 0.05$), paired t -test comparing responses in the same piece of nerve with and without antagonist.

3.3.3.3: Determining specificity of tools

The next step was to investigate the specificity of the tools for the TRPV4 ion channel. In order to do this, the TRPV4 specific ligands were initially tested in vagal tissue from *Trpv4*^{-/-} mice (**Figure 3.7**). The ligands were tested alongside agonists for the TRPV1 (capsaicin) and TRPA1 (acrolein) ion channels, as these are both known activators of vagal sensory nerves (Grace *et al.*, 2012). Channel knockout was determined using standard genotyping techniques, and an example gel is shown in **Figure 3.6** where WT bands are at 740bp and *Trpv4*^{-/-} at 1490bp.

In the knockout mice, responses to the TRPV4 ligands GSK101 and 4αPDD were virtually abolished (**Figure 3.7** $p < 0.05$). Responses to the TRPV1 ligand capsaicin (1μM) and the TRPA1 ligand acrolein (300μM) remained unchanged in both wild type and knockout tissue. In addition there was no change in any of the responses to the endogenous prostanoid agonists PGE₂ and PGD₂, which are also known to activate vagus tissue (Maher *et al.*, 2009).

3. Investigating a role for TRPV4 in airway sensory nerves

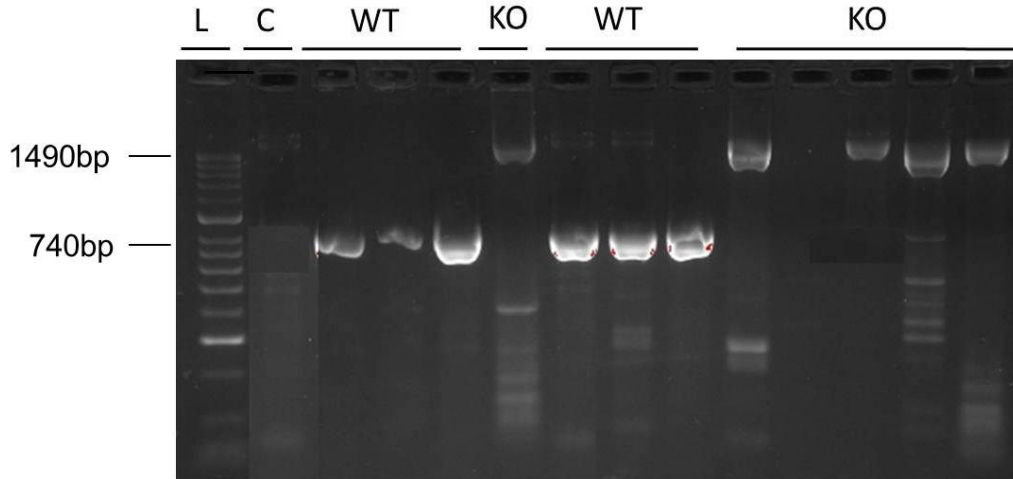


Figure 3.6: *Trpv4*^{-/-} gel

RT-PCR was used to confirm knockout of the TRPV4 gene using DNA extracted from tail tips from mice used in **Figure 3.7**. Wild type mice produced a band at 740bp and KOs at 1490bp. A 50-2000bp ladder was used (L), and a water control (C). Wild type and knockout primers were run in the same reaction.

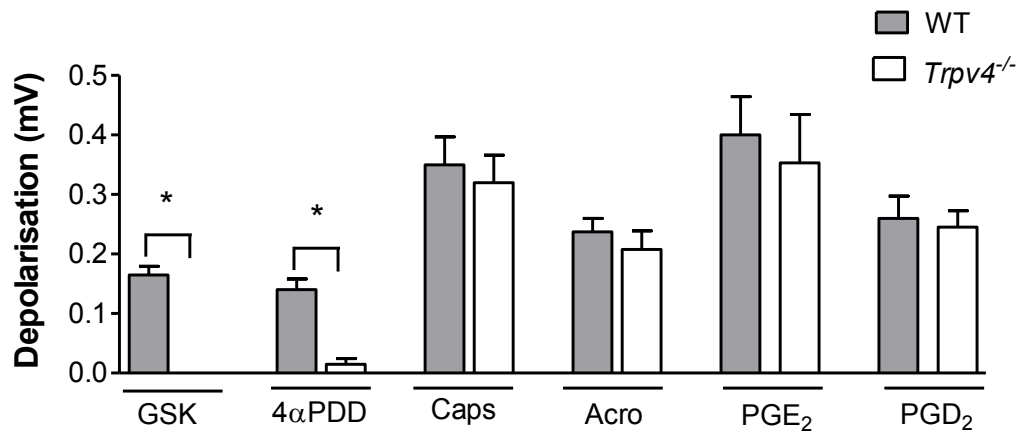


Figure 3.7: TRP ligands in *Trpv4*^{-/-} mice

Responses to the selective TRPV4 ligands GSK101 (300nM) and 4αPDD (1μM) were abolished in *Trpv4*^{-/-} mice. Responses to the TRPV1 ligand Capsaicin (1μM), the TRPA1 ligand Acrolein (300μM) and the endogenous prostanoids PGE₂ (10μM) and PGD₂ (10μM) were unaltered. The data is presented as mean ± S.E.M.. of n=4-6. * indicates statistical significance ($p < 0.05$), unpaired t-test comparing responses in *Trpv4*^{-/-} tissue with wild-type control.

As the responses were found to be abolished in the knockout tissue, the ligands were then pharmacologically evaluated against TRPV1 and TRPA1 selective antagonists to ensure that the

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agonists were not having an off target effect on the TRPV1 and TRPA1 ion channels. Depolarisation by GSK101 (300nM) and 4αPDD (1μM) were tested against the TRPA1 selective antagonist HC030031 (10μM, (Grace *et al.*, 2012)) and the TRPV1 selective antagonist JNJ17230212 (100μM; (Grace *et al.*, 2012)), using HC067047 (10μM) as a positive control in both guinea pig and mouse tissue (**Figure 3.8**). Depolarisation was inhibited by the specific TRPV4 antagonist HC067047, but the TRPA1 antagonist (HC030031; 10μM) or TRPV1 antagonist (JNJ17230212; 100μM) had no effect on GSK101 (300nM) or 4αPDD (1μM) induced depolarisation in either guinea pig (**A,B**) or mouse (**C,D**) tissue (**Figure 3.8**).

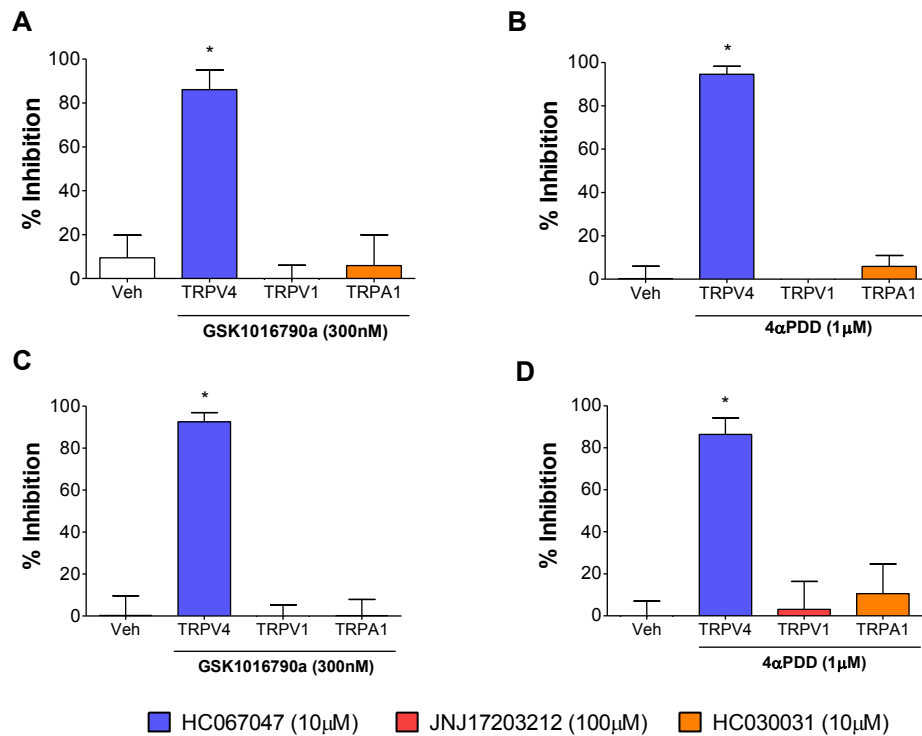


Figure 3.8: Effect of TRPA1 and TRPV1 antagonists on depolarisation induced by TRPV4 agonists. The TRPV4 ligands GSK101 and 4αPDD were tested against the selective TRPA1 and TRPV1 antagonists HC030031 (10μM) and JNJ17203212 (100μM) in both guinea pig (A,B) and mice (C,D). In both species, both GSK101 and 4αPDD were inhibited by the TRPV4 antagonist HC067047 (10μM), but neither the TRPA1 antagonist nor the TRPV1 antagonist had any effect on depolarisation induced by either agonist. Data are presented as mean ± S.E.M. of n=4-6 observations. * indicates statistical significance ($p < 0.05$), paired *t*-test comparing responses in the same piece of nerve with and without antagonist.

The specificity of the TRPV4 antagonists was also evaluated against depolarisation induced by capsaicin (1μM; TRPV1) and acrolein (300μM; TRPA1) in guinea pig and mouse tissue (**Figure**

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3.9). Neither antagonist had any effect on capsaicin nor acrolein induced depolarisation, indicating that the antagonists had no effect on either TRPV1 or TRPA1 in guinea pig (**A-B**) or mouse tissue (**C-D**) and were therefore suitable tools to take forward *in vivo*.

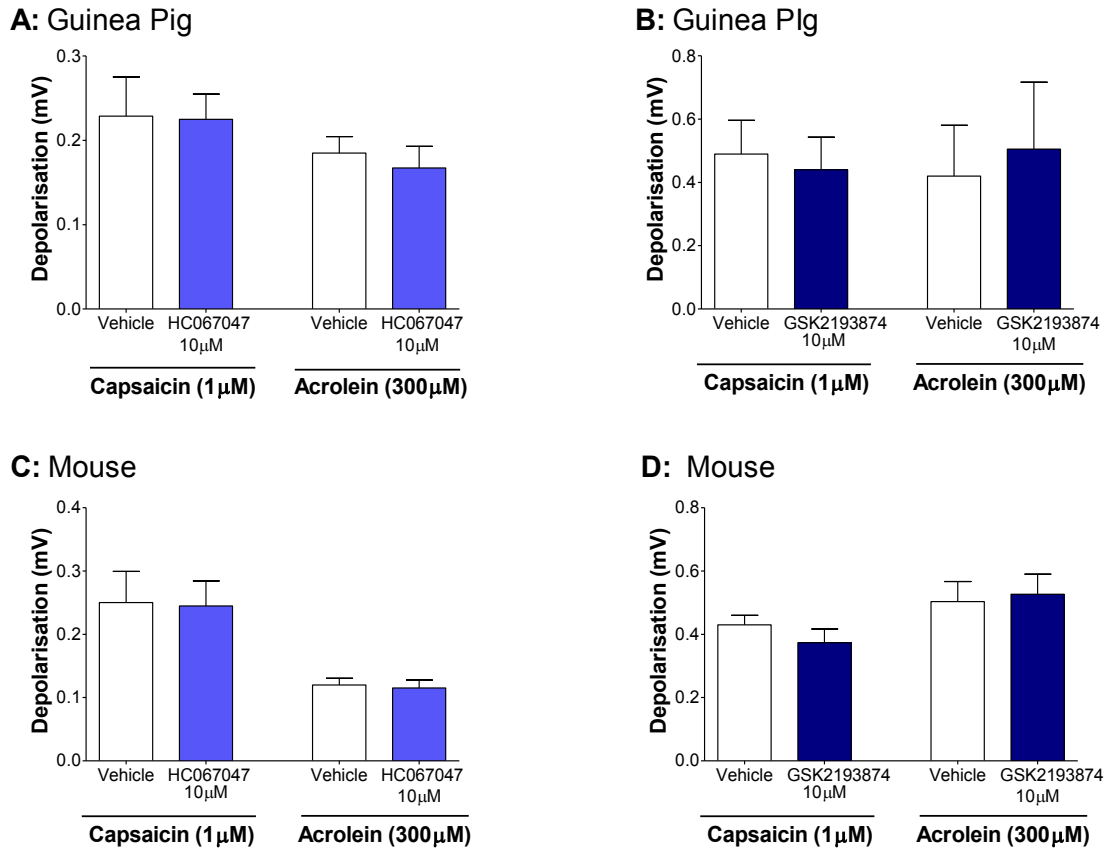


Figure 3.9: Effect of TRPV4 antagonists on TRPV1 and TRPA1 induced depolarisation

Neither HC67047 (10 μM) (A) nor GSK2193874 (10 μM) (B) had any effect on TRPV1 (Capsaicin) or TRPA1 (Acrolein) induced depolarisation in the guinea pig. A similar result was seen in mouse tissue (C,D). The data is presented as mean ± S.E.M of n=3-6 observations

3.3.3: Effect of TRPV4 agonists on firing of single vagal fibres *in vivo*

Although TRPV4 ligands have been shown to cause induction of Ca^{2+} and depolarisation of the vagus, this may not be indicative of inducing an action potential. This was investigated using the *in vivo* single fibre recording technique which allows the investigation of action potential firing from a single airway terminating afferent fibre. In addition, this technique provides a method to probe which airway specific fibre is responsible for TRPV4 mediated activation of the vagus nerve.

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The effect of aerosolised GSK101 (10µg/ml, 1 minute) was investigated on both C-fibres and Aδ-fibres. These fibres were distinguished according to capsaicin and citric acid sensitivity, response to hyperinflation and deflation and spontaneous activity, and ultimately through determination of conduction velocity (CV) (Adcock *et al.*, 2003). The 3 C-fibres under investigation had CVs of 0.52-0.92imp/s, and the 3 faster conducting Aδ-fibres had CVs of 2.3-7.14imp/s. Two of the 3 Aδ-fibres were capsaicin sensitive.

GSK101 (10µg/ml) caused a significant increase in firing in all 3 Aδ-fibres under investigation, but had no effect on C-fibres (**Figure 3.10 A**). The frequency of impulses was increased from 2.0 ± 0.6 imp/s to 12.4 ± 3.9 imp/s following application of GSK101 in the Aδ-fibres (**Figure 3.10 B**), whereas there was no increase in the C-fibres investigated following GSK101, however both capsaicin and citric acid both caused a significant increase in firing frequency (**Figure 3.10 C**). Firing induced by GSK101 was much slower in onset to that caused by capsaicin or citric acid, but it was longer lasting, continuing for over 30 minutes. GSK101 also caused a marked bronchoconstriction in all animals investigated, which was also slow in onset and occurred after Aδ-fibre activation (**Figure 3.10 D**).

In order to test specificity of fibre firing induced by the TRPV4 agonist, the TRPV4 antagonist GSK2193874 (300mg/kg) was tested against GSK101 (100ng/ml) induced firing in Aδ fibres. A lower concentration of GSK101 was used as the initial concentration did not allow repeated measurements. Following i.p administration of GSK2193874, firing frequency induced by GSK101 (100ng/ml) was significantly reduced. Firing was reduced from 20.33 ± 2.848 imp/s to 5.67 ± 0.33 imp/s (**Figure 3.10 E**).

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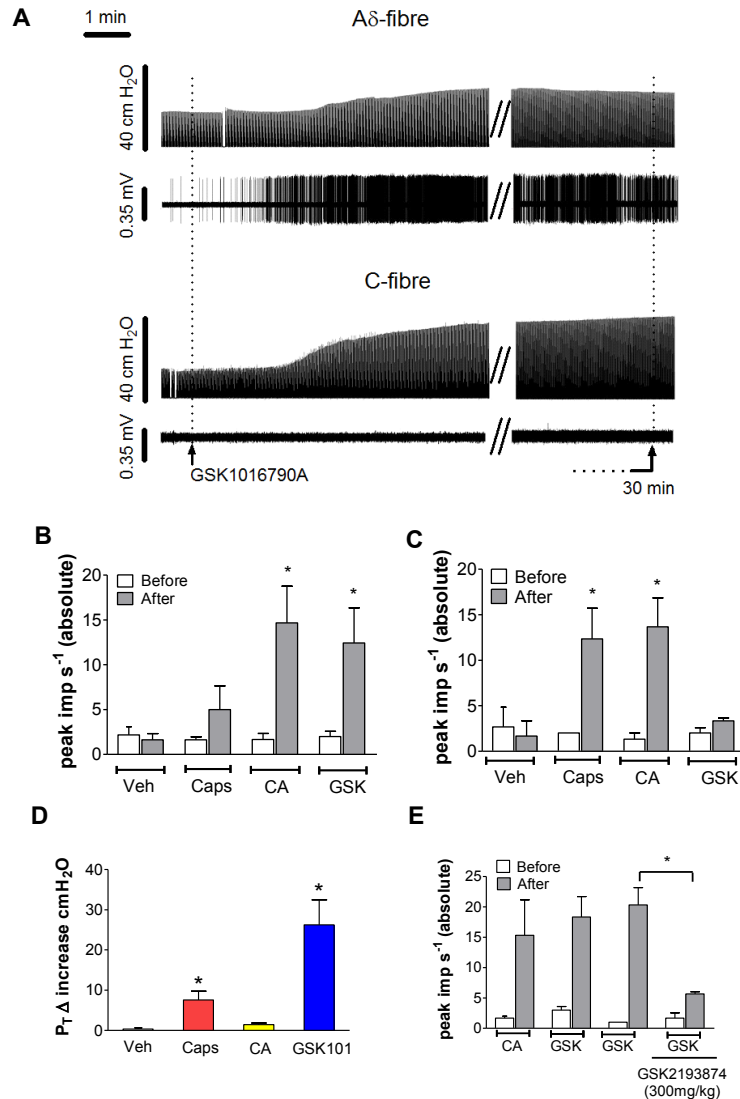


Figure 3.10: Effect of aerosolised GSK101 on single fibre firing *in vivo*

(A): Aerosolised GSK101 (10µg/ml, 1 minute) caused firing in Aδ fibres, but not C fibres, and also caused a marked bronchoconstriction as indicated by the example trace. The top panel of each trace indicated increase in tracheal pressure (increase in cmH₂O) following application of the agonist, and the lower panel indicates action potential firing. (B): Both citric acid (0.3M) and GSK101 caused a significant increase in firing frequency in Aδ fibres, but only capsaicin (100µM) and citric acid caused an increase in C fibres (C). (D): GSK101 (10µg/ml) agonist caused a large sustained bronchoconstriction in all animals. (D: Aδ fibres investigated and C fibres not shown). (E): The TRPV4 antagonist GSK2193874 (300mg/kg in 6% Cavitron/2-Hydroxypropyl-β-cyclodextrin in saline v/v) significantly inhibited firing induced by GSK101 (100ng/ml) in Aδ fibres. The data is presented as mean ± S.E.M. of n=3 observations. Veh = vehicle. * indicates statistical significance ($p < 0.05$), paired t test comparing responses before and after aerosol administration of agonist or antagonist.

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3.3.4: Effect of the TRPV4 agonist GSK1016790a on the naïve guinea pig cough response

GSK101 has been shown to activate the vagal ganglia and depolarise vagal nerves, and cause firing of A δ -fibres, which are all indicative of a cough response *in vivo*. However, the ability of a TRPV4 agonist to cause reflex cough in a conscious whole animal system is yet to be determined. Therefore a guinea pig conscious cough model was next used to investigate the tussive capability of the more potent of the two TRPV4 ligands; GSK101.

Aerosolised GSK101 (1-30 μ g/ml) caused a dose dependent increase in number of coughs in naïve, conscious guinea pigs (**Figure 3.11 A**). The type of coughs caused by the agonists included single explosive coughs similar to those seen with acrolein and capsaicin (Grace *et al.*, 2012) but also caused trains of smaller coughs, similar to those seen with PGE₂ (Maher *et al.*, 2009). Aerosolised vehicle (1% ETOH and 1% TWEEN in saline) had no effect on cough.

A submaximal concentration of the GSK101 agonist (3 μ g/ml) was then taken forward to test against the TRPV4 specific antagonist HC067047 (100mg/kg i.p.). The antagonist caused a significant inhibition in the number of coughs caused by GSK101 compared to vehicle control (**Figure 3.11B**). The alternate antagonist GSK2193874 (300 mg/kg i.p.) reduced the number of coughs caused by the highest concentration of GSK101 (30 μ g/ml) (**Figure 3.11 C**). As these were two separate experiments with different cohorts of guinea pigs, a higher concentration of GSK101 was required for the second experiment to induce a similar tussive response. Vehicle for either antagonist had no effect on the cough response.

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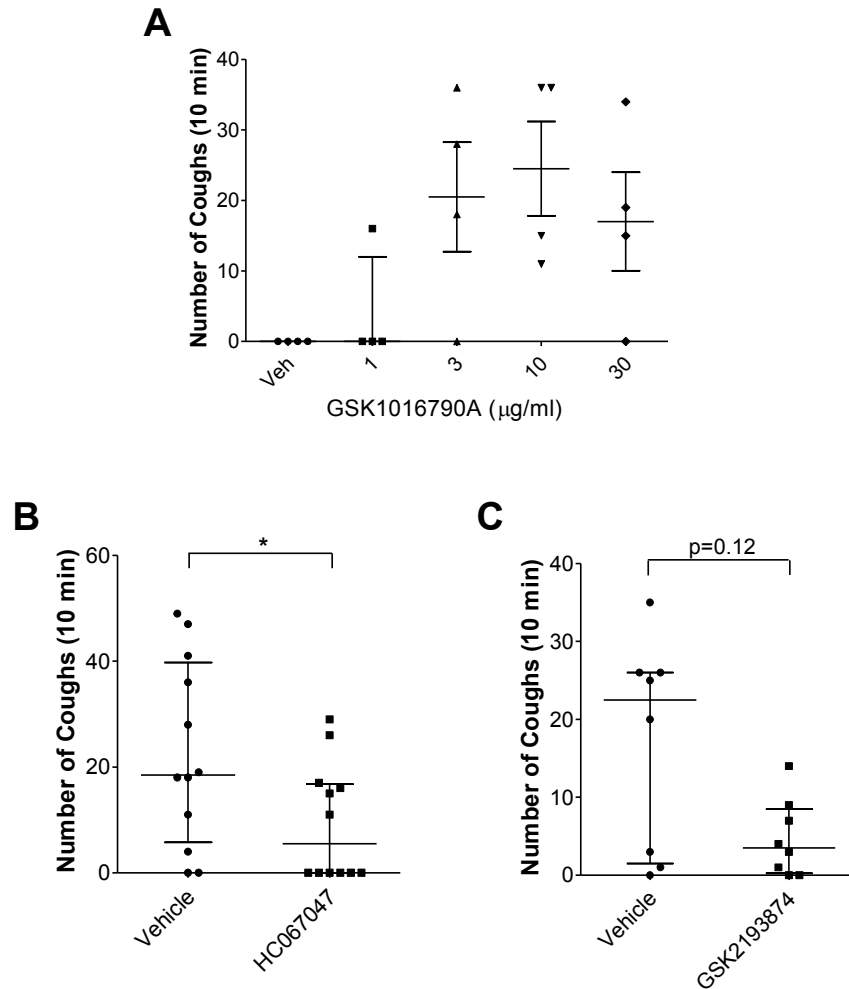


Figure 3.11: GSK101 induced cough

(A): GSK101 (aerosolised for 5 minutes) caused a dose dependent increase in number of coughs in conscious, naïve guinea pigs. (B) The selective TRPV4 antagonist HC067047 (100mg/kg in 1% DMSO in 0.9% saline v/v i.p administered 1 hour before GSK101 exposure) significantly inhibited the number of coughs induced by GSK101 (3 $\mu\text{g/ml}$). (C) An alternate TRPV4 antagonist GSK2193874 (300mg/kg i.p.) also inhibited the number of coughs induced by GSK101 (30 $\mu\text{g/ml}$, 5 minutes), but did not reach significance. Vehicle (0.1% DMSO and 1% TWEEN in saline) had no effect on the cough response. The data is presented as median $\pm$ interquartile range of (A) $n=4$, (B) $n=12$ and (C) $n=8$ observations. * indicates statistical significance ($p < 0.05$), Mann Whitney U test comparing responses from the antagonist group to vehicle control.

3. Investigating a role for TRPV4 in airway sensory nerves

3.4: Discussion

The aim of this chapter was to investigate a role for the TRPV4 ion channel in activation of airway sensory nerves and cough. There is currently an abundance of literature that outline the role of two TRP channels TRPV1 and TRPA1 in airway sensory nerve activation and cough (for example; (Andr  *et al.*, 2009; Birrell *et al.*, 2009; Grace *et al.*, 2012), however the role of TRPV4 remains relatively unexplored. TRPV4 is a calcium permeable ion channel which has been shown to be expressed at the mRNA level in pulmonary sensory neurons in rats (Ni *et al.*, 2006). A role for TRPV4 in the airways has been eluded to as TRPV4 ligands are known to cause contraction of airway smooth muscle (Jia *et al.*, 2004; McAlexander *et al.*, 2014) and polymorphisms of the channel are present in COPD (Zhu *et al.*, 2009). TRPV4 is activated by a number of endogenous ligands present in the diseased airway such as arachidonic acid derivatives (Watanabe *et al.*, 2003) and an altered airway osmolarity (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Jia *et al.*, 2004), where airway sensory nerve responses are thought to be altered, and therefore it may be assumed that TRPV4 could play a role in airway sensory nerve activation and cough. This was evaluated by determining a functional role for TRPV4 in airway ganglia and in depolarisation of vagal nerves, in addition *in vivo* studies were carried out to investigate the effect of TRPV4 on airway single fibre firing and guinea pig conscious cough.

Before determining a functional role for TRPV4, it was established that the TRPV4 ion channel was expressed at the mRNA level in nodose and jugular ganglia, which contain the cell bodies of the afferent fibres which project to the lung. As TRPV4 was expressed in both ganglia, a functional role was determined using calcium imaging of airway specific fibres. It was shown that GSK101, a potent and selective activator of the TRPV4 ion channel (Thorneloe *et al.*, 2008), caused an increase in $[Ca^{2+}]_i$ signal in airway specific nodose ganglia in a concentration dependent manner. Interestingly, in all cases there appeared to be a short delay prior to activation. However GSK101 did not appear to affect the jugular ganglia tested. In general, it is thought that the more chemosensitive C-fibres originate from the jugular ganglia, whereas the more mechanosensitive A -fibres originate from the nodose (Ricco *et al.*, 1996; Undem *et al.*, 2004; Canning *et al.*, 2006b), therefore suggesting a potential novel role for TRPV4 in activation of mechanosensitive A -fibres.

The effects of TRPV4 ligands were then pharmacologically evaluated in the *in vitro* model of vagal nerve depolarisation. Both guinea pig and mouse tissue were used as guinea pigs have a cough reflex similar to that of man, and mice, as although they do not cough per se, the afferent arm of the reflex has been shown to act similarly (Maher and Belvisi, 2010), and also allows the use of

3. Investigating a role for TRPV4 in airway sensory nerves

transgenic animals. The selective TRPV4 ligands GSK101 and 4αPDD were shown to cause concentration dependent increases in depolarisation in both guinea pig, and mouse tissue. The submaximal concentration of GSK101 taken forward for antagonist studies was 300nM, and 4αPDD; 1μM in the guinea pig tissue, which relate to the published pEC50 values from mouse of 7.7 for GSK101 and 6.7 for 4αPDD (Watanabe *et al.*, 2002; Thorneloe *et al.*, 2008). The selective TRPV4 antagonists HC067047 and GSK2193874 were both shown to inhibit submaximal responses of each of these agonists indicating that the depolarisation seen was through activation of the TRPV4 ion channel. Both antagonists elicited maximal inhibition at 10μM, which would indicate that the antagonists are less potent than the published IC50s (HC067047 of 7.8 and GSK219 of 8.3 in mouse tissue) would suggest (Everaerts *et al.*, 2010; Thorneloe *et al.*, 2012). This could signify that these antagonists are less potent in guinea pig tissue, or differences in the *in vitro* preparation used.

A major advantage of the vagus technique meant that key experiments with both agonists and antagonists could be repeated in donor human tissue. TRPV4 ligands were shown to activate human vagal nerves, and this activation was inhibited by the selective antagonists to a similar degree as seen in rodent tissue. This indicates that the results obtained with guinea pig and mouse tissue are likely to translate to the clinic.

Selectivity of the ligands was determined initially in TRPV4 knockout mice, where the responses to both GSK101 and 4αPDD were abolished in the knockout tissue; however responses to the TRPV1 and TRPA1 agonists capsaicin and acrolein, and the endogenous prostanoid agonists PGE₂ and PGD₂ remained unchanged. This highlights a further difference between TRPV4 and the further characterised TRP channels; depolarisation induced by PGE₂ has been shown to be due to indirect agonism of both the TRPV1 and TRPA1 ion channels (Grace *et al.*, 2012), whereas it was clear that PGE₂ induced responses were unchanged in the TRPV4 knockouts. Furthermore, both GSK101 and 4αPDD induced depolarisation was tested against the TRPV1 selective antagonist JNJ17203212 and the TRPA1 antagonist HC030031 where it remained unaffected. Neither HC067047, nor GSK2193874, showed any inhibitory effect on capsaicin or acrolein induced depolarisation in the guinea pig or mouse. This therefore indicates that the TRPV4 ligands used were selective for their receptor.

By using *in vitro* studies to pharmacologically evaluate the TRPV4 ligands, this provided a reliable alternative to *in vivo* experiments which can have complex pharmacokinetic issues and require a large number of animals and compounds, and are therefore also expensive. However, the vagus

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nerve can also innervate visceral organs other than the airways (Standring, 2005), and also the expression of receptors and signal transduction pathways on the nerve trunk may not be the same as on the nerve endings (Patel *et al.*, 2003). Therefore to circumvent this, the more potent of the two ligands, GSK101, was taken forward into *in vivo* studies where its ability to propagate an action potential, and also cause cough in the conscious guinea pig was evaluated.

According to current understanding there are several known airway sensory afferents as described in the introduction ([Section 1.3.1](#)). Of these, three fibre types are more mechanically sensitive; RARs (classical RARs, irritant receptors and A δ fibres (Adcock *et al.*, 2003, 2014)), SARs and the cough receptor. Chemosensitive afferent fibres include the A δ nociceptors and C fibres. GSK101 was shown to cause sustained firing (lasting for over 30 minutes) of the A δ fibre subset of RARs (CVs ranging from 2.3-7.14m/s) in the anaesthetised guinea pig, and of the 3 fibres investigated, two were capsaicin sensitive. The ligand did not induce firing of any of the C fibres investigated. In addition this firing was significantly inhibited following i.p. administration of the TRPV4 antagonist GSK2193874 (300mg/kg). The A δ fibres under investigation had a spontaneous discharge with a clear rhythmical respiratory pattern and adapted rapidly to hyperinflation and deflation. These fibres were a subset of the fast conducting, myelinated RAR fibres which conduct action potentials in the A δ range (CV >3m/s (Canning *et al.*, 2006b)), are thought to be housed in the nodose ganglia and respond to mechanical stimuli along with citric acid (Ricco *et al.*, 1996; Adcock *et al.*, 2003; Canning *et al.*, 2006b). In addition it has recently been shown that there is a subset of RAR A δ fibres which respond to capsaicin regardless of conduction velocity (Adcock *et al.*, 2014). In contrast, a large number of chemical stimuli which have been shown to cause cough in animals and in man along with TRPV1 and TRPA1 ligands, have been shown to more commonly activate the chemosensitive C fibres (Coleridge and Coleridge, 1984; Laloo *et al.*, 1995; Canning, 2004; Birrell *et al.*, 2009). These fibres are unmyelinated and slower conducting (CV <2m/s), are relatively insensitive to mechanical stimuli and are thought to be mostly derived from the jugular ganglia (Undem 2004, Canning 2006). As the fibres are unresponsive to mechanical stimuli, the chemical stimuli activate the nerves directly (Undem 2004; Chuaychoo *et al.* 2005).

RARs are traditionally thought to be extremely sensitive to mechanical stimuli, but are not typically activated by a direct chemical stimulus (with the exception of citric acid) and therefore activation of a typically mechanosensitive fibre by a TRPV4 ligand is a highly interesting and novel observation. RARs can be indirectly activated following bronchoconstriction and mucus production induced by activation of C fibres (Widdicombe, 2003; Canning *et al.*, 2006a); however

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in all cases with TRPV4, bronchoconstriction began after fibre firing, so activation is unlikely to be through this mechanism. Alongside the Ca^{2+} data from the nodose ganglia, these results confirm a novel role for TRPV4 in activation of mechanosensitive A δ fibres. This novel observation, along with the mechanism of activation requires further investigation.

It was interesting to note that TRPV4 was expressed at the mRNA level in both the nodose and the jugular ganglia, but a functional response was only seen in the nodose and in A δ fibres; GSK101 did not seem to have an effect on the calcium signal from the jugular ganglia or on C fibre firing. This would suggest that TRPV4 is present on C fibres, but activation does not lead to action potential firing. However, mRNA expression does not indicate protein levels and active channel activity, so in order to further examine this, protein levels should be measured from both ganglia. Unfortunately the lack of reliable antibodies for TRP channels has meant that protein level could not be assessed at this time. One caveat to mention is that expression was measured in whole ganglia, which houses a variety of cell types including dendritic cells, glia and macrophages. Therefore TRPV4 may not be expressed on nerves themselves and instead on surrounding cells which could then induce signalling pathways which lead to activation of sensory nerves.

In addition to prolonged fibre firing, GSK101 caused a marked, sustained bronchoconstriction in all animals investigated. The sustained firing could have been a secondary result of this bronchoconstriction seen with GSK101, as it occurred on mechanosensitive A δ fibres. However, it has been shown *in vitro* that GSK101 is capable of causing compound depolarisation of the vagus nerve and alter $[\text{Ca}^{2+}]_i$ signal in the vagal ganglia, in systems free from bronchoconstriction. In addition, firing began prior to bronchoconstriction in all fibre types investigated, and in all cases, bronchoconstriction was slow in onset and lasted for over 30 minutes. Furthermore, both vagal nerves were cut at the central end and so this response seems unlikely to be through a reflex event. Previous work *in vitro* has indicated that GSK101 is able to cause contraction of isolated guinea pig and human airway smooth muscle (Jia *et al.*, 2004; McAlexander *et al.*, 2014), through release of cysteinyl leukotrienes, which was blocked by TRPV4 antagonists. These results would suggest that TRPV4 is able to cause bronchoconstriction independent from A δ fibre firing.

Although the single fibre technique allows the investigation of airway fibre firing, as both vagal nerves were cut and the guinea pig anaesthetised, it was yet to be seen if TRPV4 was able to cause a full reflex event in a conscious animal. In order to investigate this, a guinea pig cough model was utilised which allowed the aerosolisation of compounds to the conscious guinea pig

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where cough responses could be monitored by two blinded trained cough observers, and also effects on bronchoconstriction could be monitored. The key advantage of this technique is that studies have shown that tussive responses induced in guinea pigs closely resemble that seen in man (Laude *et al.*, 1994; Birrell *et al.*, 2009), and therefore results could translate to the clinic. GSK101 was demonstrated to cause a concentration dependent increase in the cough response in the conscious guinea pig. The cough response was inhibited by two antagonists of TRPV4; HC067047 and GSK2193874. These results indicate that GSK101 is capable of causing full reflex events in a conscious animal through the TRPV4 channel.

In summary, TRPV4 ligands have been shown to directly activate airway sensory nerves *in vitro*, through causing an increase in $[Ca^{2+}]_i$ signal in airway cells from the nodose ganglia, and also causing concentration dependant depolarisation in both guinea pig and human vagal nerves. *In vivo*, GSK101 was shown to cause a significant, sustained firing of mechanosensitive A δ fibres, and also caused reflex cough in the conscious guinea pig. This body of data outlines a role for the TRPV4 ion channel in activation of sensory nerves and the cough reflex, through activation of nodose derived A δ mechanoreceptors, a different mechanism to what is seen with other TRP channels. Therefore, it can be concluded that the chapter hypothesis 'Activation of TRPV4 on airway sensory nerves by selective agonists leads to nerve activation and cough' can be accepted. This is an exciting and novel observation which requires further investigation.

Throughout this chapter the role of TRPV4 has been determined using exogenous, synthetic ligands which, although are selective for the TRPV4 ion channel, do not occur *in vivo*. TRPV4 was originally characterised as a sensor of osmotic stress, (Strotmann *et al.*, 2000; Liedtke and Friedman, 2003; Suzuki *et al.*, 2003) as *Trpv4*^{-/-} mice show abnormalities with systemic osmotic and external somatosensory stimuli (Liedtke *et al.*, 2000) and also as a mechanosensor, as a sensor of osmotic stress it is sensitive to cell swelling (Liedtke *et al.*, 2000). Airway osmolarity is known to be altered in disease (Joris *et al.*, 1993; Jia *et al.*, 2004), where airway sensory nerve responses are known to be enhanced. Therefore in the following chapter, I will outline a role for the activation of sensory nerves by TRPV4 using changes in osmolarity; an endogenous, disease relevant stimuli.

Chapter 4:

The role of TRPV4 in
osmotic activation of airway
sensory nerves

4: The role of TRPV4 in osmotic activation of airway sensory nerves

4.1: Rationale

Alterations in airway osmolarity can occur in disease; in asthmatics the osmolarity airway surface fluid in the lungs is decreased (Joris *et al.*, 1993), and asthmatics also experience exercise induced bronchoconstriction following dehydration of the airway surface liquid after exercise which leads to an increase in airway osmolarity (Weiler *et al.*, 2010; Anderson and Kippelen, 2012; Hallstrand, 2012). Furthermore, solutions of both increased and decreased osmolarity have been reported to cause activation of airway sensory nerves and reflex events such as cough in both animals and humans (Eschenbacher *et al.*, 1984; Fuller and Collier, 1984; Laloo *et al.*, 1995). However, the mechanism by which this occurs in airway sensory nerves is yet to be determined. The ion channel TRPV4 was originally characterised as a sensor of osmotic stress, and mice lacking the TRPV4 ion channel have shown abnormalities in osmotic regulation (Liedtke, *et al.*, 2000; Mizuno *et al.*, 2003; Suzuki *et al.*, 2003). In addition, TRPV4 has been well documented to act as an osmosensor and mechanosensor in both nerves and the brain (Alessandri-Haber *et al.*, 2003; Mizuno *et al.*, 2003; Suzuki *et al.*, 2003). As demonstrated in the previous chapter, activation of TRPV4 is capable of causing firing of airway sensory nerves and cough in a conscious animal, and therefore it could be hypothesised that TRPV4 acts as an osmosensor in airway sensory nerves.

4.1.1: Chapter Hypothesis:

TRPV4 channels present on airway sensory nerves are activated by changes in osmolarity

4.1.2: Aims;

- This will initially be achieved by pharmacological characterisation of solutions of both increased (prepared using increasing concentrations of sucrose) and decreased (prepared by salt reduction and compensation with sucrose) osmolarity. These solutions will be evaluated using the *in vitro* isolated vagus system with guinea pig, murine and human tissue
- To determine the role of TRPV4 and also TRPV1 and TRPA1 in the activation of sensory nerves by changes in osmolarity, using selective antagonists of the TRPV1 (JNJ17203212; 100µM), TRPA1 (HC030031; 10µM) and TRPV4 (HC067047; 10µM and GSK2193874; 10µM) channels alongside *Trpv1*^{-/-}, *Trpa1*^{-/-} and *Trpv4*^{-/-} knockout mice.
- Finally the solutions will then be taken forward to be tested *in vivo* to determine whether they are able to propagate an action potential in guinea pig single fibres, and if so which fibre type is responsible.

4.2: Methods

4.2.1: Preparation of solutions

Hyperosmotic solutions were prepared fresh on the day of the experiment using a modified Krebs Heinslett solution with increasing concentrations of sucrose. Modified Krebs solution was prepared as described previously in [Chapter 2](#), and to increase the osmolarity of the solution, increasing concentrations of sucrose were added. A solution of 305mOsm (10 mOsm above normal osmolarity of ~295mOsm (Denton *et al.*, 1996; Bourque and Oliet, 1997)) was prepared by adding 10mM of sucrose (+10mOsm), a solution of 325mOsm; 30mM (+30mOsm), a solution of 395mOsm; 100mM (+100mOsm), a solution of 495mOsm; 200mM (+200mOsm) and a solution of 595mOsm; 300mM (+300mOsm). The control solution for these experiments was modified Krebs solution.

The hypoosmotic solutions were also made fresh on the day of the experiment using a modified Krebs-Heinslett solution with salt reduction, followed by gradual compensation with sucrose. A solution of 195mOsm (100mOsm below normal osmolarity) contained (in mM; 7 KCl, 63 NaCl, 1.2 MgCl₂, 1.2 NaH₂PO₄, 5.6 D-Glucose, 25.5 NaHCO₃, 2.5 CaCl₂). Solutions of increasing osmolarity were then made by addition of sucrose. For solutions of 215mOsm (-80), 235mOsm (-60), 255mOsm (-40), 275mOsm (-20) and 295mOsm (0), sucrose was added to the modified Krebs solution (in mM: -80mOsm: 20, -60mOsm: 40, -40mOsm: 60, -20mOsm: 80, 0mOsm: 100). As the chloride content of these solutions is significantly reduced and therefore could have a functional effect, the 0mOsm solution was used both before and after application of the hypoosmotic solution.

Osmolarity of all solutions used was confirmed using a cryo-osmometer (Osmomat030, Gonotec, Germany).

4.2.2: Isolated vagal nerve recordings

Vagal tissue was dissected from male Dunkin-Hartley guinea pigs, WT C57/Bl6 mice, and knockout (*Trpv4*^{-/-}, *Trpa1*^{-/-} and *Trpv1*^{-/-}) mice, and placed in a grease-gap recording chamber as previously described in [Section 2.6](#). Human donor tissue was also obtained for translational data, where key experiments were repeated.

4.2.2.1: Concentration response curves

Concentration response curves were carried out in WT C57/Bl6 mice, male Dunkin Hartley guinea pigs and donor human vagal tissue. In order to determine if osmotic solutions were able to depolarise the vagal nerve, the following experimental protocol was undertaken. Non-

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cumulative concentrations of either hypoosmotic (0mOsm to -100mOsm below normal osmolarity of 295mOsm), or hyperosmotic solutions (0mOsm to +1000mOsm above normal osmolarity of 295mOsm) were applied to the vagal nerve trunk followed by a wash period where the nerve was washed with Krebs solution until it returned to baseline. Responses were recorded as mV depolarisation. A submaximal concentration of each of the osmotic stimuli was then taken forward to be tested against TRP antagonists and in knockout mice.

4.2.2.2: Pharmacological assessment of hyper and hypo-osmolarity induced depolarisation: Knockout Mouse

In order to determine the role of TRP channels in depolarisation caused by increases or decreases of osmolarity, initially mice devoid of the *Trpv4*, *Trpv1* or *Trpa1* gene were used in the *in vitro* isolated vagus nerve system. Although mice do not have a functional cough response, the afferent arm of the reflex has been reported to act similarly and the use of knockouts allows the investigation of the importance of a particular receptor in a response without pharmacological intervention. Knockout of the *Trpv1* or *Trpa1* gene was confirmed using standard genotyping techniques as described in [Section 2.3](#). Responses to both hyper- and hypoosmotic solutions were established in *Trpv4*^{-/-}, *Trpv1*^{-/-} and *Trpa1*^{-/-} tissue, and in age matched wild type controls. As a control measure, responses to capsaicin (1μM; TRPV1 agonist) acrolein (300μM; TRPA1 agonist) and GSK101 (300nM; TRPV4 agonist) were also established in each of the knockouts and wild type tissue.

4.2.2.3: Pharmacological assessment of hyper and hypo-osmolarity induced depolarisation: Guinea pig and human

Depolarisation induced by either hyperosmotic or hypoosmotic solutions was then tested against specific antagonists for the TRPV1, TRPA1 and TRPV4 channels in guinea pig tissue and translational results obtained in donor human vagus. Selective antagonists for TRPV1 (JNJ17203212; 100μM), TRPA1 (HC030031; 10μM) and TRPV4 (HC067047; 10μM and GSK2193874; 10μM) were pharmacologically evaluated against depolarisation induced by a submaximal concentration of hyper- or hypoosmotic solution.

A classical pharmacological approach was used; two reproducible responses to each osmotic solution was established, following which each nerve was incubated with a single concentration of one of the antagonists for ten minutes. Following incubation, the nerve was restimulated with agonist in the presence of antagonist to determine inhibition. The nerve was then washed with Krebs until returned to baseline, and a final response to the agonist carried out to ensure nerve viability.

4.2.2.4: Analysis

Individual nerve depolarisations were measured in mV, and the chart recorder calibrated so that 1mm was equal to 0.01mV. Antagonist inhibitions were calculated by comparing the magnitude of depolarisation of the nerve following antagonist incubation to the mean magnitude of the two control agonist responses, to give a % inhibition. Statistical analysis for inhibition studies was carried out in guinea pig and WT mouse tissue using a two tailed t-test for matched pairs comparing the magnitude of depolarisation by the agonist with and without the presence of antagonist in the same piece of nerve. For mouse knockout work, an unpaired t-test was used to compare responses between WT and KO mice. For concentration responses, the data was analysed using a Kruskal Wallis test and Dunns post-test comparing responses to vehicle. The data is presented as mean $\pm$ S.E.M., with statistical significance set at $P < 0.05$.

4.2.3: Genotyping of knockout mice

The genotyping details for TRPV4 have been outlined previously in [Chapter 3](#). Homozygous breeding pairs of mice genetically modified to disrupt the TRPV1 (*Trpv1*^{-/-}) and TRPA1 (*Trpa1*^{-/-}) genes were purchased from Jackson Laboratories (USA) (Caterina, 2000; Kwan *et al.*, 2006). Breeding colonies were maintained on a C57Bl/6 background at Imperial College London and regular genotyping was carried out to ensure continued deletion of the TRPV1 and TRPA1 allele. This involved amplification of the DNA sequence of interest using PCR as previously described in [Section 2.3.1](#).

DNA samples extracted from the tail tips of WT (C57Bl/6), *Trpv1*^{-/-} and *Trpa1*^{-/-} mice were amplified by PCR using primers (Invitrogen) and visualised on an agarose gel by electrophoresis. Samples were heated to 94°C, following which 40 cycles of denaturing, annealing and extension were carried out, the exact duration and temperatures of which are outlined in **Table 4.1**. The resulting PCR products were run on 2% agarose gel (80V for 1 hour) in TBE buffer containing 0.05 μ l/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) and a DNA ladder (Hyperladder, Bioline Reagents, UK). For TRPV1, the expected wild type product was 984bp and knock out 600bp and for TRPA1, wild type product expected was 317bp and knockout 184bp.

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Table 4.1: PCR Protocol for *Trpv1*^{-/-} and *Trpa1*^{-/-} mice

	PCR Conditions			
TRPV1	Denaturing 95°C 30 sec	Annealing 64°C 1 min	Extension 72°C 1 min	Product Size (Base Pairs) WT: 984bp KO: 600bp
TRPA1	Denaturing 95°C 30 sec	Annealing 68°C 30 sec	Extension 72°C 1 min	Product Size (Base Pairs) WT: 317bp KO 184bp

4.2.3: *In vivo* single fibres

Male Dunkin Hartley guinea pigs (~350-400g) were anaesthetised with urethane (1.5g/kg i.p.) and paralysed with vecuronium bromide (0.1mg/kg i.v.) and artificially ventilated. Heart rate, blood pressure and body temperature were constantly monitored for the duration of the experiment. Guinea pigs were then prepared as described in [Section 2.7.1](#) and as previously described (Adcock *et al.*, 2003). Briefly, both vagal nerves were cut at the cervical end but only the left vagal nerve used for recording. This 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. The nerve fibre was identified as originating from the family of SARs, RARs or chemosensitive C fibres using a set of criteria (Adcock *et al.*, 2003) and as described in [Section 2.7.3](#). This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, Adaptation Indices, response to capsaicin and citric acid and finally conduction velocity which was determined at the end of the experiment.

4.2.3.2: Agonist responses

The following experimental protocol was undertaken to determine the effect of hypoosmolarity on airway terminating vagal fibres. Following surgery, the animals were left to stabilise for 30 minutes, following which a control baseline recording was taken for 2 minutes. Depending on fibre type, responsiveness to capsaicin (C fibre: 100µM) or citric acid (Aδ fibre: 300mM) was determined and changes in fibre activity, intra-tracheal pressure and blood pressure were constantly monitored until they returned to baseline or a steady state achieved. Following this, a 0mOsm solution was administered for 1 minute by aerosol and variables were continuously recorded. Following an interval of 20 min, increasing concentrations of hypoosmotic solution of (0mOsm to -100mOsm) were administered by aerosol for 1 minute, 20 minutes apart and the changes in fibre activity, intratracheal pressure and blood pressure were continuously

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recorded. Nerve viability at the end of the experiment was assessed using capsaicin or citric acid.

4.2.3.3: Analysis

The data is expressed as mean $\pm$ S.E.M. and analysed using paired students t-test comparing responses as absolute values after stimulus to baseline values immediately before the response.

4.3: Results

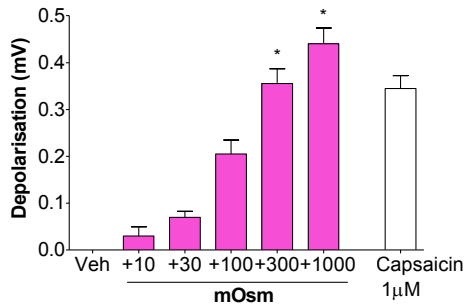
4.3.1: Effect of osmotic solutions on depolarisation of isolated vagal nerves

Initially, the ability of a hyperosmotic solution (made using modified Krebs solution and increasing concentrations of sucrose) and a hypo-osmotic solution (prepared using a modified Krebs solution with salt reduction and gradual compensation with sucrose) to depolarise isolated vagus nerves from both guinea pig and wild type mice was investigated. In addition, translational data was obtained with human tissue where available.

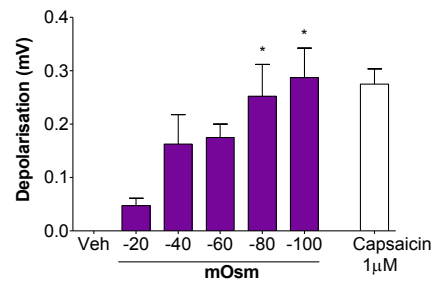
Both hyperosmotic and hypoosmotic solutions caused a concentration dependent depolarisation of guinea pig (**Figure 4.1 A,B**), mouse (**Figure 4.1 C,D**) and human tissue (**Figure 4.1 E,F**). In mice, maximum depolarisation induced by a +1000mOsm hyperosmotic solution was $0.44\text{mV} \pm 0.03\text{mV}$, in guinea pigs maximum depolarisation was $0.573\text{mV} \pm 0.104\text{mV}$ and in human tissue depolarisation maximum depolarisation was $0.260\text{mV} \pm 0.08\text{mV}$. In all cases, depolarisation induced by a +1000mOsm solution exceeded that of the control capsaicin response ($1\mu\text{M}$). For hypoosmotic solutions, the maximum depolarisation elicited by -100mOsm in mice was $0.29\text{mV} \pm 0.06\text{mV}$, in guinea pigs was $0.22\text{mV} \pm 0.05\text{mV}$ and in human tissue was $0.11\text{mV} \pm 0.01\text{mV}$. Once again, depolarisation induced by this level of hypoosmolarity exceeded that of the control capsaicin response ($1\mu\text{M}$).

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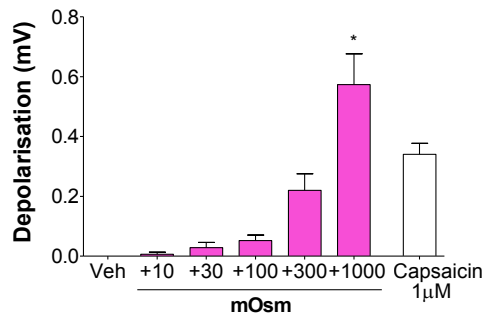
A: Mouse



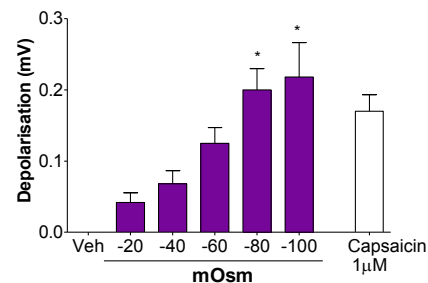
B: Mouse



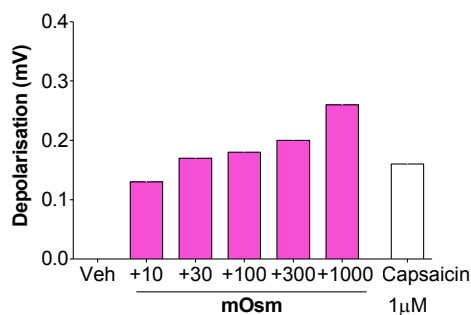
C: Guinea Pig



D: Guinea Pig



E: Human



F: Human

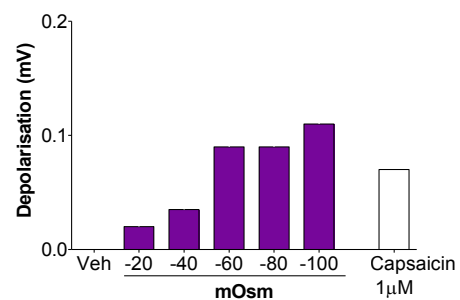


Figure 4.1: Concentration response curves to osmotic stimuli

Hyperosmotic and hypoosmotic solutions caused a concentration dependent increase in depolarisation in mouse (A,B), guinea pig (C,D) and human (E,F) tissue. Capsaicin was used in all cases as a positive control. Data is shown as mean $\pm$ S.E.M of $n=4-6$ for rodent and $n=2$ for human tissue. Human tissue was from 1 male and 1 female donor aged 38-68 with no known respiratory diseases.

** indicates statistical significance at $p<0.05$ using Kruskal Wallis test and Dunns post test comparing responses to vehicle. Due to limited N numbers, statistical analysis was not carried out on data in human tissue.*

4.3.2: Pharmacological assessment of hyper and hypo-osmolarity induced depolarisation: Knockout Mouse

In order to determine whether any TRP channels, in particular TRPV4, are involved in the depolarisation induced by osmotic changes, each solution was initially profiled in knockout mice. Although mice do not have a functional cough response it has been shown that the afferent arm of the reflex acts similarly and compounds which cause depolarisation in guinea pig and human tissue also cause depolarisation in mouse tissue. Mice are a useful model as the genetic template can be modified and knockouts can be utilised which allows the investigation of the importance of a particular receptor without pharmacological intervention.

Pairs of mice genetically modified to remove the *Trpv1*, *Trpa1* or *Trpv4* gene were obtained and bred in house, and male homozygous knockout mice used for experiments. Regular genotyping was undertaken to ensure that the gene of interest was knocked out. An example gel for the *Trpa1*^{-/-} mice and *Trpv1*^{-/-} mice is shown below (**Figure 4.2 A,B**), and an example *Trpv4*^{-/-} knockout gel was shown previously in Chapter 3. All mice were bred on a C57Bl/6 background, and therefore male C57Bl/6 mice were used as wild type controls.

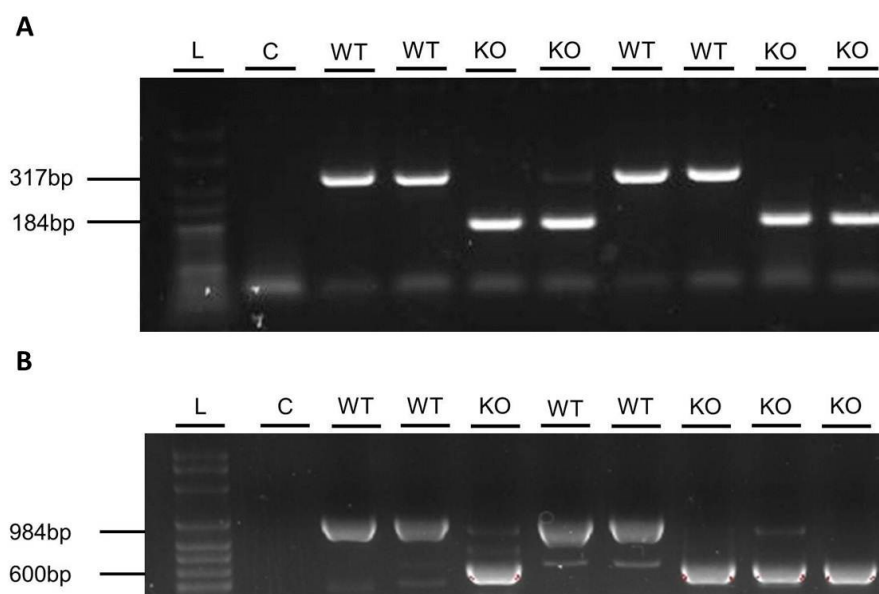


Figure 4.2 Example knockout gels

A: Trpa1^{-/-} knockout gel. RTPCR was used to confirm knockout of the TRPA1 gene using DNA extracted from tail tips from mice used in Figure 4.3. Wild type mice produced a band at 317bp and KOs at 184bp. A 25-500bp ladder was used (L), and a water control (C). Wild type and knockout primers were run in the same reaction.

B: Trpv1^{-/-} knockout gel. RTPCR was used to confirm knockout of the TRPV1 gene using DNA extracted from tail tips from mice used in Figure 4.3. Wild type mice produced a band at 984bp and KOs at 600bp. A 50-1000bp ladder was used (L), and a water control (C). Wild type and knockout primers were run in the same reaction.

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A submaximal concentration of hyperosmolar (+300mOsm) and hypoosmolar (-80mOsm) solutions were taken forward to test in the knockout mice, along with a submaximal concentration of TRPV1, TRPA1 and TRPV4 agonists: capsaicin, (1 μ M; TRPV1), acrolein (300 μ M; TRPA1) and GSK101 (300nM; TRPV4).

In vagal tissue from *Trpa1*^{-/-} mice, acrolein induced depolarisation was abolished, confirming knockout of the channel and indicating that the TRPA1 gene was not functional. Capsaicin and GSK101 induced depolarisation remained unchanged in knockout tissue indicating that loss of TRPA1 had no effect on TRPV1 or TRPV4 responses. +300mOsm induced depolarisation also remained unchanged from wild type to knockout, with depolarisation of 0.232mV $\pm$ 0.029mV in the wild type and 0.2440mV $\pm$ 0.051mV in the knockout, indicating that the TRPA1 channel does not play a role in hyperosmolarity induced depolarisation in the mouse. Knockout of the TRPA1 gene also had no effect on the -80mOsm induced depolarisation, where depolarisation of 0.225mV $\pm$ 0.06mV in the wild type mice was only slightly reduced to 0.1960 $\pm$ 0.02551mV in the knockout tissue (**Figure 4.3 A**).

In tissue from *Trpv1*^{-/-} mice, capsaicin induced depolarisation was abolished indicating that the ion channel was non-functional. Both acrolein and GSK101 induced depolarisation remained unchanged in the knockout tissue, indicating that knockout of the TRPV1 ion channel had no effect on TRPA1 or TRPV4 responses. +300mOsm induced depolarisation was only slightly reduced in the knockout tissue, with a depolarisation of 0.276mV $\pm$ 0.05mV in wild type tissue and 0.200 $\pm$ 0.035mV in knockout tissue demonstrating that the TRPV1 channel plays a minor role in hyperosmotic induced depolarisation. However, responses to -80mOsm solution were significantly reduced to around 50% in the knockout tissue, with a depolarisation of 0.3320mV $\pm$ 0.0484mV in wild type tissue reduced to 0.15mV $\pm$ 0.021mV in knockout tissue, indicating that the TRPV1 channel plays a role in hypoosmotic induced nerve depolarisation in the mouse (**Figure 4.3 B**).

In tissue from *Trpv4*^{-/-} mice, GSK101 induced depolarisation was virtually abolished, indicating that the TRPV4 channel was not functional. Acrolein and capsaicin induced depolarisation remained unchanged; indicating that knockout of the TRPV4 gene does not affect other TRP channel responses. +300mOsm induced depolarisation was unchanged in *Trpv4*^{-/-} tissue, with depolarisation of 0.16mV $\pm$ 0.025mV in the wild type tissue and 0.21 $\pm$ 0.026mV in the knockout tissue, demonstrating that the TRPV4 ion channel does not play a role in hyperosmotic induced depolarisation in the mouse. However, similarly to the *Trpv1*^{-/-} tissue, -80mOsm induced depolarisation was significantly reduced to around 50% (from 0.3050 $\pm$ 0.017mV in WT tissue to 0.1452 $\pm$ 0.017mV in the knockout) indicating that the TRPV4

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ion channel plays a major role in hypoosmotic induced depolarisation in the mouse (**Figure 4.3 C**).

This data indicates that the TRPV1 and TRPV4 ion channels are required for hypoosmotic induced depolarisation in the mouse, with the TRPA1 channel not playing a role. However, although responses to hyperosmotic stimuli were reduced in *Trpv1*^{-/-} mice, indicating that the TRPV1 ion channel may play a role, knockout of any of the TRP genes did not have any statistically significant effect on hyperosmotic induced depolarisation.

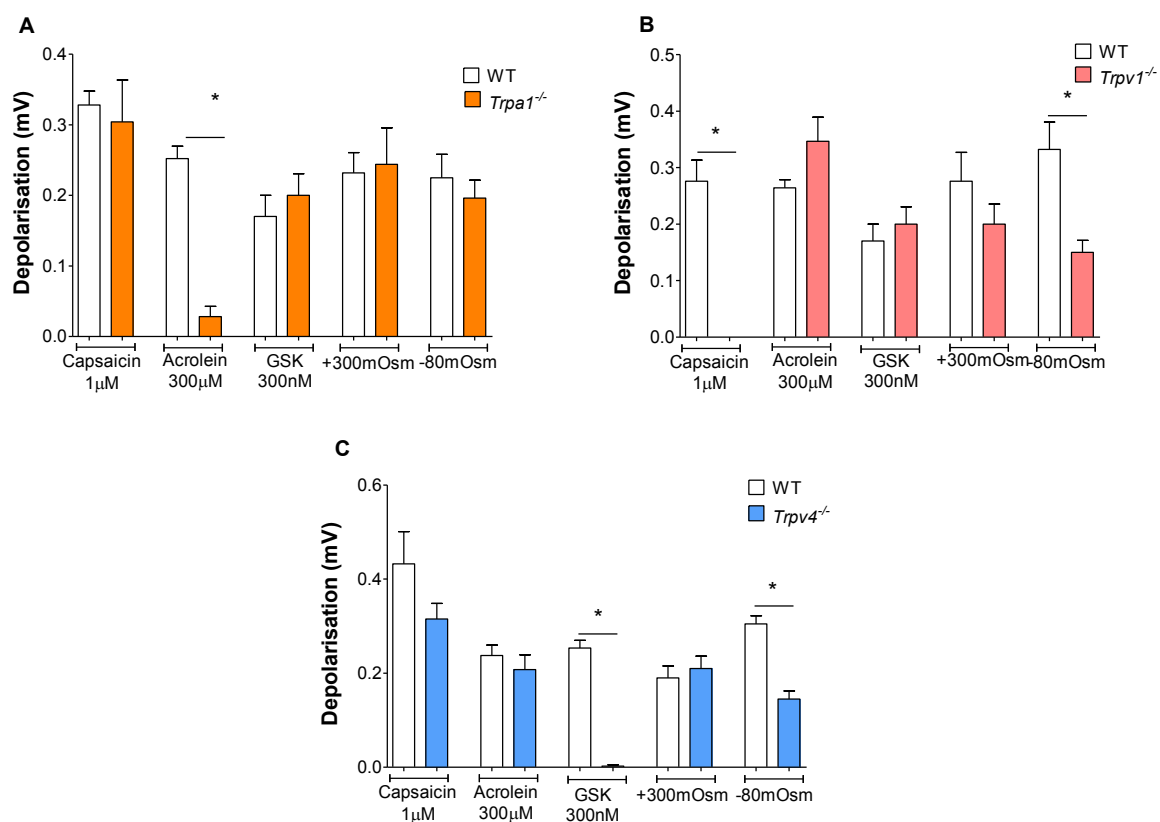


Figure 4.3: Effect of hyper and hypoosmotic solutions in knockout mice

A: *Trpa1*^{-/-} mice: Only responses to the TRPA1 agonist acrolein were significantly inhibited in the *Trpa1*^{-/-} mice. Responses to hyper (+300mOsm) and hypoosmotic (-80mOsm) solutions along with responses to TRPV1 (capsaicin) and TRPV4 (GSK101) ligands remained unchanged. **B:** *Trpv1*^{-/-} mice; Responses to capsaicin and hypoosmotic solutions were significantly inhibited in the *Trpv1*^{-/-} mice and responses to hyperosmotic solution were reduced. However responses to TRPA1 agonists and TRPV4 agonists remained unchanged. **C:** *Trpv4*^{-/-} mice: Responses to GSK101 and hypoosmotic solutions were significantly reduced in the *Trpv4*^{-/-} mice. Responses to hyperosmotic solutions along with TRPV1 and TRPA1 agonists remained unchanged. The data is presented as mean $\pm$ S.E.M.. of n=4-6. * indicates statistical significance ($p < 0.05$), unpaired t-test comparing responses in knockout tissue with wild-type control.

4.3.3: Pharmacological assessment of hyper and hypo-osmolarity induced depolarisation: Guinea Pig and Human vagal tissue

Although the knockout mice provided key data without pharmacological intervention, as mouse airway innervation differs to that of guinea pigs and humans and as such may possess a different confirmation of receptors and fibres. Therefore, osmotic stimuli were assessed against specific TRP antagonists in both guinea pig and donor human vagal nerve tissue, which are more physiologically relevant systems. The osmotic stimuli were pharmacologically assessed against specific antagonists for the TRPV1 (JNJ17203212: 100µM), TRPA1 (HC030031: 10µM) and TRPV4 (HC067047: 10µM and GSK2193874: 10µM) ion channels (**Figure 4.4**). A summary of the inhibition induced by each of the antagonists is shown in **Table 4.2**.

For depolarisation induced by hyperosmotic solutions, in the guinea pig the TRPV1 antagonist significantly inhibited the response $80.9 \pm 9.4\%$, whereas TRPA1 and TRPV4 seemed to play a similar smaller role in inhibiting depolarisation induced by the osmotic stimulus, both inhibiting the response 30-50%. In contrast in human tissue, TRPA1 and TRPV4 both played a bigger role in inhibition of the response; both antagonists inhibited the response by around 60%, whereas the TRPV1 antagonist played a much smaller role. This would indicate a species difference in the mechanism of hyperosmotic induced depolarisation (**Figure 4.4 A, B**). The results seen in the guinea pig tissue were similar to those seen in the knockout mouse, where only tissue from *Trpv1*^{-/-} showed any reduction in depolarisation induced by hyperosmotic solution.

For depolarisation induced by hypoosmotic solution, a similar profile was seen in both guinea pig and human tissue. In both cases, the TRPV4 antagonist HC067047 was shown to have the greatest inhibition of hypoosmotic induced depolarisation, with HC067047 inhibiting the response $86.33\% \pm 12.30\%$ in guinea pigs and $76.95\% \pm 3.74\%$ in human tissue. GSK2193874 also inhibited the response similarly in guinea pig tissue. The TRPV1 antagonist also played a large role, inhibiting the response by around 50% in both species. TRPA1 was shown to have less of an effect (**Figure 4.4 C,D**). This is concurrent with the knockout mouse data, where depolarisation induced by -80mOsm was significantly inhibited in both *Trpv1*^{-/-} and *Trpv4*^{-/-} mice.

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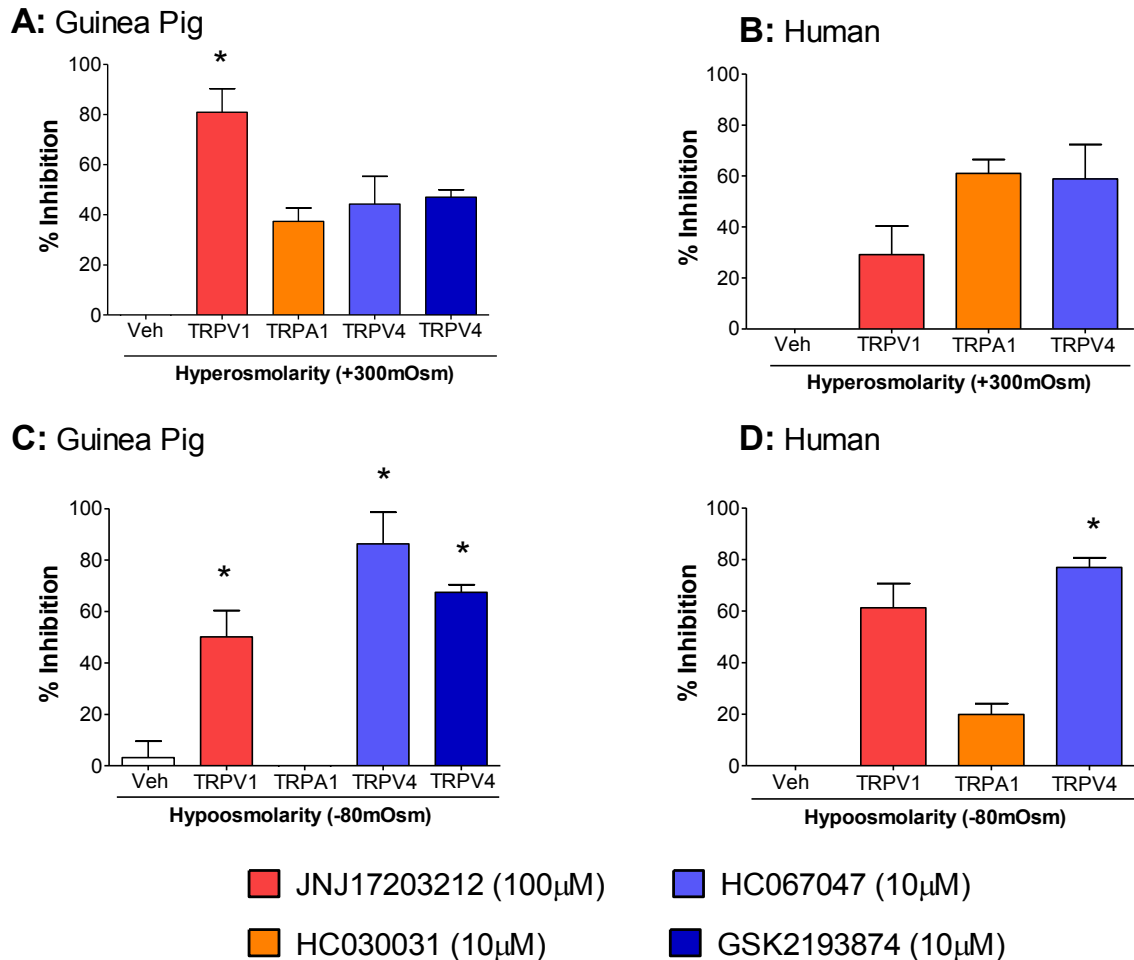


Figure 4.4: Effect of TRP antagonists on osmotic activation of vagal nerves

A: Guinea pig: The TRPV1 antagonist JNJ17203212 (100μM) significantly inhibited depolarisation induced by hyperosmotic solution (+300mOsm), the TRPA1 (HC030031: 10μM) and TRPV4 antagonists (HC067047: 10μM and GSK2193874: 10μM) all inhibited the response similarly to around 30-40%. **B: Human:** Both TRPA1 and TRPV4 antagonists inhibited the responses to around 50-60%, however the TRPV1 antagonist seemed to play a smaller role only inhibiting the response to around 30%. **C: Guinea pig:** Both the TRPV1 antagonist and the TRPV4 antagonists significantly inhibited the depolarisation induced by hypoosmotic solution (-80mOsm), with the TRPA1 antagonist having no effect. **D: A similar profile was seen in donor human tissue.** *n*=4 for GP and *n*=3 for human. Human tissue was obtained from 2 female and 1 male donor aged 38-68 with no known respiratory disease. * indicates statistical significance (*p* < 0.05), paired *t*-test comparing responses in the same piece of guinea pig nerve with and without antagonist. Due to limited numbers of human experiments, statistical analysis was not undertaken.

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Table 4.2: Summary of the role of TRP channels in depolarisation induced by osmotic solutions.

ANTAGONIST	GUINEA PIG	HUMAN
<i>Hyperosmolar Solutions (+300mOsm)</i>		
JNJ17203212 (100µM: TRPV1)	80.9±9.4%	29.1±11.2%
HC030031 (10µM: TRPA1)	37.3±5.4%	62±5.5%
HC067047 (10µM: TRPV4)	27.1±18.8%	58.9±13.5
GSK2193874 (10µM: TRPV4)	47±2.9%	N/A
<i>Hypoosmolar Solution (-80mOsm)</i>		
JNJ17203212 (100µM: TRPV1)	50.1±10.20%	61.29±9.39%
HC030031 (10µM: TRPA1)	0%	19.9±4.3%
HC067047 (10µM: TRPV4)	86.3±12.3%	76.9±3.7%
GSK2193874 (10µM: TRPV4)	67.4±2.9%	N/A

N/A: Antagonist not tested.

4.3.4: Characterisation of hypo osmotic solution on firing of single vagal fibres *in vivo*

As depolarisation induced by hypoosmotic solution was found to be predominantly through activation of TRPV4, and because of limited time, only hypoosmotic solution was taken forward *in vivo* to test in the airway single fibre recording technique. This technique allows the investigation of action potential firing from a single airway terminating afferent fibre, in the absence of a full reflex arc as both vagal nerves are cut at the central end.

A concentration response to aerosolised hypoosmotic solution (0mOsm to -100mOsm below normal osmolarity) was carried out on both C-fibres and Aδ-fibres. These fibres were distinguished according to their capsaicin and citric acid sensitivity, response to hyper-inflation and deflation, spontaneous activity and ultimately through determination of a conduction velocity (CV) at the end of the experiment. The 3 C-fibres under investigation had CVs of 0.47-0.85imp/s and a total of 4 Aδ fibres were investigated with CVs of 4.7-11.3imp/s. Two of the 4 Aδ-fibres investigated responded to capsaicin.

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Citric acid activated all A δ fibres under investigation, and in all cases a solution of 0mOsm below normal airway osmolarity was used as a control. Hypoosmotic solutions caused a concentration dependent increase in firing frequency in the A δ fibres examined, but solutions with an osmolarity of -60mOsm, -80mOsm and -100mOsm below normal osmolarity increased fibre firing significantly in the A δ fibres investigated. -60mOsm increased firing frequency from 1imp/s to 11.75 ± 2.056 imp/s, -80mOsm increased firing frequency from 2.33 ± 0.33 imp/s to 10.33 ± 1.764 imp/s and -100mOsm increased firing from 1.67 ± 0.67 imp/s to 11.33 ± 1.76 imp/s (**Figure 4.5A**). All fibres responded to citric acid at the end of the experiment.

Capsaicin activated all C fibres under investigation and a solution of 0mOsm was used as a control. In contrast to the A δ fibres investigated, hypoosmotic solution did not cause a concentration dependent increase in firing in C fibres. Instead, only a solution of -100mOsm increased firing frequency significantly from 0 to 19 ± 4.933 imp/s (**Figure 4.5B**). All fibres responded to capsaicin at the end of the experiment.

In all cases, hypoosmotic solution caused bronchoconstriction, which was significant at -80 and -100 mOsm, as indicated by an increase in tracheal pressure (**Figure 4.5C,D**).

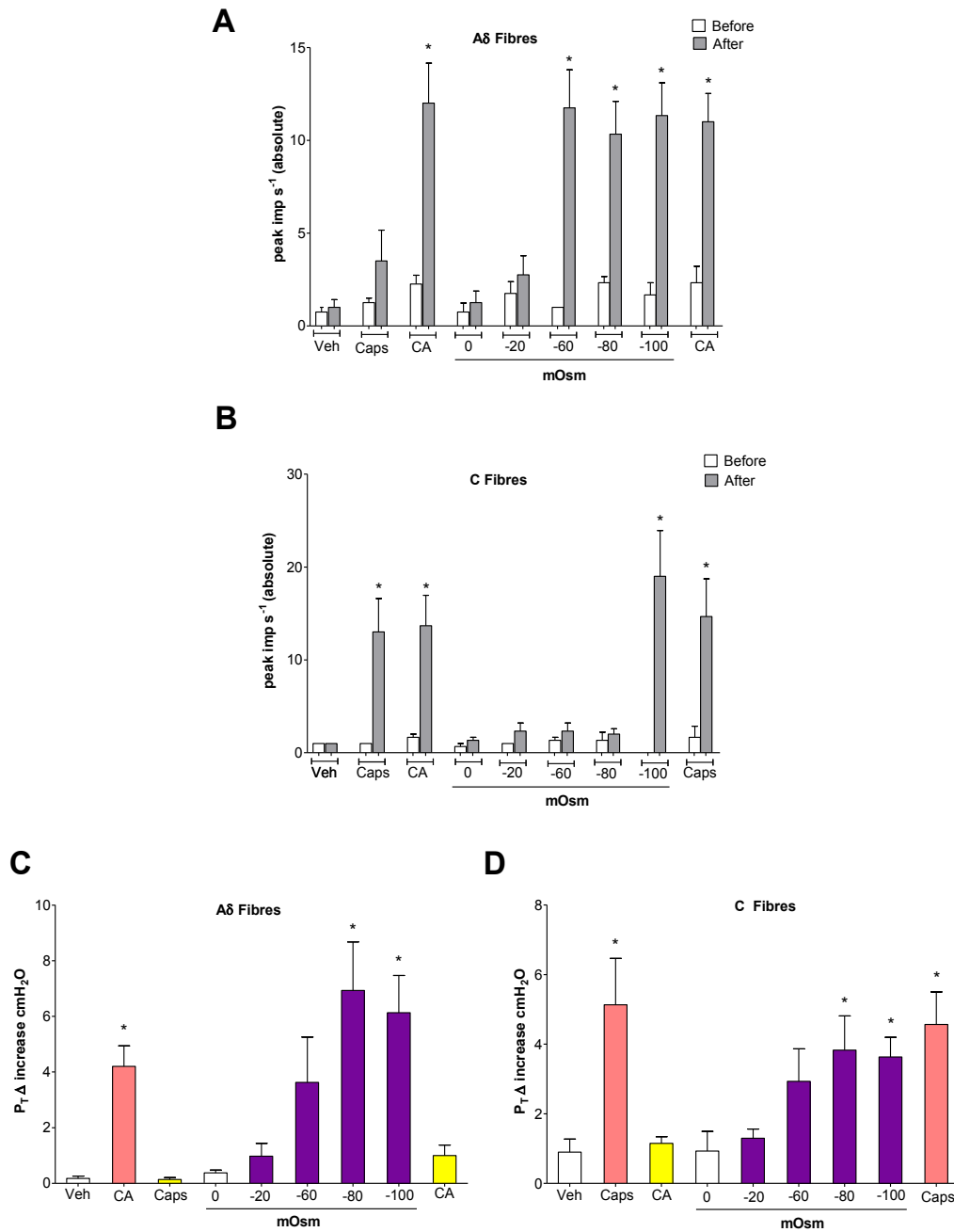


Figure 4.5: Effect of osmolarity on firing of airway sensory nerves

(A): Aδ-fibres: Aerosolised hypoosmotic solution (0mOsm to -100mOsm) caused a concentration dependent increase in firing frequency in Aδ-fibres, with -60mOsm to -100mOsm causing a significant increase. Citric acid also caused a significant increase in firing frequency. (B): C-fibres: Both capsaicin and citric acid caused a significant increase in firing frequency, but only hypoosmotic solution at -100mOsm was able to activate the C-fibres examined. (C,D). C and D indicate that hypoosmotic solution caused a large sustained bronchoconstriction in all animals (C: Animals where Aδ-fibres were investigated and D: animals where C-fibres were investigated). The data is presented as mean ± S.E.M. of n=3-4 observations. Veh = vehicle. * indicates statistical significance ($p < 0.05$), paired t test comparing responses before and after aerosol administration of agonist.

4.4: Discussion

The aim of this chapter was to determine the mechanism of osmotic activation of airway sensory nerves, and to determine whether TRPV4 plays a role. Solutions of both increased and decreased osmolarity have been reported to cause activation of airway sensory nerves and reflex events such as cough in both animals and humans (Fox *et al.*, 2005.; Eschenbacher *et al.*, 1984; Fuller and Collier, 1984; Lalloo *et al.*, 1995). Hypoosmotic solution is well documented to be an activator of the ion channel TRPV4 (Alessandri-Haber *et al.*, 2003; Jia *et al.*, 2004) and TRPV4 is activated by changes in osmolarity as little as -30mOsm (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000). TRPV4 has also been shown to be the ion channel transducer which mediates nociceptive behaviour induced by small changes in osmolarity (Alessandri-Haber *et al.*, 2005). However, the role of TRPV4 in osmotic activation of airway sensory nerves is yet to be determined. Therefore in this chapter, the role of TRPV4 as an osmosensor in airway sensory nerve was explored by investigating activation of vagal nerves induced by both hyper and hypoosmotic stimulation, and using both specific TRPV4 antagonists alongside tissue from TRPV4 knockout mice. The role of other TRP channels TRPV1 and TRPA1 was also investigated as these have been shown to play a role in airway sensory nerves, and along with TRPV4, TRPV1 has also been shown to contribute to osmosensitivity of neurons (Bonini *et al.*, 1996; Alessandri-Haber *et al.*, 2003).

Osmotic stimulation is defined as a deviation from an osmotic set point, which in many animals is $\sim 295\text{mOsm l}^{-1}$ (Denton *et al.*, 1996; Bourque and Oliet, 1997). The osmolarity of the airways is determined by the airway surface fluid, which is a low viscosity thin layer covering the mucosal surface of the airway epithelia which line the conducting airways (Joris *et al.*, 1993). In normal, healthy humans, the osmolarity of airway surface fluid is thought to be mildly hypoosmotic, with an osmolarity of 222mOsm, which is around 80% of the isotonic body fluids (Joris *et al.*, 1993). In asthmatic patients, the osmolarity of airway surface fluids is thought to be further reduced (Joris *et al.*, 1993). At a cellular level, hypoosmolarity is a mechanical stimulus, as it causes cells to swell which is a process dependent upon Ca^{2+} channels (Lang *et al.*, 1998). The hypoosmotic solution distilled water is a potent activator of airway fibres *in vivo* (Fox *et al.*, 1995) and ultra-nebulised distilled water is used as a cough challenge agent in the clinic (Eschenbacher *et al.*, 1984; Dicipinigaitis, 2007). This is reported to cause both cough and bronchoconstriction in subjects (Cheney and Butler, 1968; Fontana *et al.*, 1999, 2002). Together with the fact that hypoosmolarity is a known activator of the TRPV4 ion channel (Jia *et al.*, 2004), it was decided to investigate the role of TRPV4 in hypoosmotic induced depolarisation.

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In this study, hypoosmotic solutions caused a concentration dependant increase in depolarisation in mouse, guinea pig and human tissue. Depolarisation in all species was found to be predominantly through the TRPV4 ion channel, as *Trpv4*^{-/-} mice and treatment with the TRPV4 antagonists HC067047 (10µM) and GSK2193874 (10µM) significantly inhibited vagal nerve activation induced by hypoosmotic solutions. However, despite blocking the majority of the response (around 70-80%) in all species, the TRPV4 antagonist did not block the whole response. The remainder of the activation instead looks to be through the TRPV1 ion channel, with TRPA1 antagonists having no effect different to that of vehicle. Both TRPV1 and TRPV4 have been shown to contribute to osmosensitivity (Bonini *et al.*, 1996; Alessandri-Haber *et al.*, 2003), where they have previously been shown to play complementary roles (Lechner *et al.*, 2011). Most importantly, translational results were obtained in human tissue, which would suggest that these results may translate to the clinic.

In vivo, hypoosmotic solutions were shown to activate Aδ-fibres, the same fibre type as activated by the TRPV4 agonist GSK101, at osmolarity changes as small as -60mOsm. However, hypoosmotic solutions were only able to activate C-fibres at an osmolarity of -100mOsm, indicating Aδ-fibres are more sensitive to activation by smaller changes in osmolarity. This agrees with previous work which has shown that distilled water (a solution of around -300mOsm) activated all Aδ-fibres in the guinea pig and dog, and also all C-fibres in the guinea pig (Pisarri *et al.*, 1992; Fox *et al.*, 1995). In all cases, solutions from -60mOsm upwards caused bronchoconstriction as indicated by an increase in tracheal pressure. This is in agreement in with data from the clinic which has shown that cough challenge with distilled water (fog) does cause bronchoconstriction as well as cough in the subjects (Cheney and Butler, 1968; Fontana *et al.*, 1999, 2002).

Responses of TRPV4 to hypotonic solutions, phorbol esters and heat have been shown to be transient with rapid decay and are dependent on calcium (Plant and Strotmann, 2007). However, TRPV4 induced activation by hypoosmotic stimuli has been shown to involve a different mechanism to activation caused by 4αPDD and heat. Hypoosmotic solutions induce cell swelling which induces activation of PLA₂ and causes the release of polyunsaturated fatty acids including arachidonic acid from membrane phospholipids. It is thought that the arachidonic acid is then broken down by the enzyme cytochrome P450 epoxygenase which produces 5,6 epoxy-eicosatrienoic acid (5,6 EET) which then activates TRPV4 (Vriens *et al.*, 2004; Vriens *et al.*, 2005). Evidence for this theory exists as four structurally unrelated inhibitors of PLA₂ along with a cytochrome p450 epoxygenase inhibitor, have been shown to block swelling induced Ca²⁺ signals in TRPV4 expressing HEK cells, but had no effect on activation of TRPV4 by heat, 4αPDD or arachidonic acid derivatives (Vriens *et al.*, 2005). In

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addition, it has recently been shown that activation of TRPV4 by hypoosmotic stimulus involves PIP₂ binding to a domain in the N terminus, and this interaction rearranges the cytosolic domains (Garcia-Elias *et al.*, 2013).

Although in normal, healthy humans the airway surface fluid is hypoosmotic, it can also become hyperosmotic, which is common during exercise where evaporation of water leads to dehydration of airway surface liquid and an increase in osmolarity (Anderson *et al.*, 2010; Anderson and Kippelen, 2012). In asthmatics this has been known to lead to exercise induced bronchoconstriction (Weiler *et al.*, 2010; Anderson and Kippelen, 2012; Hallstrand, 2012). Furthermore, hyperosmolar solutions are known to cause cough and bronchoconstriction in both humans (Eschenbacher *et al.*, 1984) and guinea pigs (Lalloo *et al.*, 1995) and the hypertonic solution mannitol (which is devoid of both sodium and chloride ions) is a commonly used tussive stimulus in the clinic (Morice *et al.*, 2007). Therefore, it was decided to also investigate the role of TRPV4 in hyperosmotic induced activation of airway sensory nerves.

Depolarisation induced by hyperosmotic solution was shown to be partly conducted through the TRPV1 ion channel, with *Trpv1*^{-/-} mice showing a reduction in depolarisation induced by a hyperosmotic stimulus of +300mOsm, and in guinea pigs, depolarisation induced by +300mOsm was significantly reduced following application of the TRPV1 antagonist JNJ17203212 (100µM). This is concurrent with the current literature where TRPV1 has already been implicated in osmolarity sensing in neurons from the Organum Vasculosum Lamina Terminalis (OVLT), which is responsible for the monitoring of blood solute concentration, in the CNS (Ciura and Bourque, 2006; Ciura *et al.*, 2011). Despite TRPV4 being the widely accepted TRP channel osmosensor, it was shown that hypertonicity induced excitation of these neurons was abolished in *Trpv1*^{-/-} mice (Ciura and Bourque, 2006), whereas *Trpv4*^{-/-} mice remained unaffected (Ciura *et al.*, 2011), and TRPV4 has previously been shown not to be activated *in vitro* by hypertonicity (Alessandri-Haber *et al.*, 2005). Furthermore, responses to both hyperosmolar stimulation and cell shrinkage were abolished in wild type mice following treatment with a TRPV1 antagonist (Ciura *et al.*, 2011). *Trpv1*^{-/-} mice have been shown to be chronically hyperosmolar and show an impaired water intake and ADH release in response to i.p. injections of hypertonic saline (Ciura and Bourque, 2006; Sharif Naeini *et al.*, 2006). Taken together this would suggest that TRPV1 is responsible for hyperosmotic induced activation of airway sensory nerves in both the mouse and the guinea pig. However, this was not the case in the translational human tissue, where both TRPV4 and TRPA1 antagonists were shown to inhibit hyperosmolarity induced depolarisation, with TRPV1 playing a lesser effect. This highlights the importance of carrying out translational work in human tissue.

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In order to expand upon the single fibre data and in order to ensure that the firing seen is through TRPV4, a TRPV4 antagonist should be taken *in vivo* to test against a submaximal concentration of hypoosmolarity on both C fibres and A δ fibres. As ultra-nebulised distilled water is used as tussive stimuli in the clinic, hypoosmotic solutions could also be taken forward into the conscious guinea pig cough system, and the role of TRPV4 assessed using antagonists. Effect of hypoosmotic solutions can also be investigated on $[Ca^{2+}]_i$ signal in isolated ganglia, to determine if hypoosmotic solutions activate the nodose ganglia similarly to the TRPV4 ligands. Alongside hypoosmotic solution, an effect of hyperosmotic solutions on fibre firing and cough in conscious guinea pigs is something that can be investigated at a later time point. Previous studies have indicated that hypertonic stimuli activate C afferents (Fox *et al.*, 1995; Pedersen *et al.*, 1998) and have induced tachykinin release from isolated cultured DRG neurons which were phenotypically C fibres (White *et al.*, 1995). Furthermore, both stimuli could also be taken forward in disease models. As the epithelium is damaged in respiratory disease (Laitinen *et al.*, 1985; Elia *et al.*, 1988), it could be hypothesised that airway sensory nerve terminals may be more exposed to the airway surface fluid which may lead to activation of the TRPV4 or TRPV1 channels, which may contribute to the enhanced reflex events seen in disease.

In summary, the chapter hypothesis 'TRPV4 channels present on airway sensory nerves are activated by changed in osmolarity' can be partially accepted. This body of data has shown that activation of airway sensory nerves by hypoosmotic solutions are predominantly mediated through the TRPV4 ion channel in all species examined. This agrees with previous data which outlines hypoosmotic solutions as activators of the TRPV4 ion channel (Alessandri-Haber *et al.*, 2003; Jia *et al.*, 2004). TRPV4 antagonists inhibited the response to -80mOsm induced depolarisation, and depolarisation was also significantly inhibited in *Trpv4*^{-/-} mice. Hypoosmotic stimuli were also found to activate both A δ and C fibres, however A δ fibres were much more sensitive to smaller changes in osmolarity, and these fibres were the same fibres as activated by the TRPV4 agonist GSK101. In contrast, activation of sensory nerves by hyperosmotic solutions is predominantly mediated through TRPV1 in murine and guinea pig tissue, with little effect of TRPV4. This is an important, novel finding as alterations of airway osmolarity occur in disease, and this data would suggest that the TRPV4 and TRPV1 channels may play a role in mediating the effects.

Chapter 5:

The role of ATP and P2X3
in TRPV4 mediated
activation of airway sensory
nerves

5: The role of ATP and P2X3 in TRPV4 mediated activation of airway sensory nerves

5.1: Rationale

In the previous chapters it has been demonstrated that activation of TRPV4 by both synthetic agonists and by hypoosmolar solutions causes depolarisation of the vagus nerve, single airway fibre firing and reflex cough in the conscious guinea pig through activation of nodose derived A δ fibres. Both calcium signal and firing of airway afferent fibres demonstrated a short delay prior to activation by the TRPV4 agonist GSK101 indicating the possible release of a secondary mediator. In the bladder, activation of TRPV4 by 4 α PDD, 5,6-EET and mechanical stretch has been shown to cause an increase of [Ca²⁺]_i and ATP release (Birder *et al.*, 2007; Mochizuki *et al.*, 2009). In addition firing of bladder afferents by the TRPV4 agonist GSK101 has been shown to be blocked by the P2X inhibitors PPADs and also the P2X3/P2X2/3 antagonist TNP-ATP (Aizawa *et al.*, 2012). Furthermore, it has recently been shown that TRPV4 plays a key role in cigarette smoke induced ATP release in human primary epithelial cells (Baxter *et al.*, 2014). As the ion channel P2X3 is expressed in both human and guinea pig airway sensory nerves (Kwong *et al.*, 2008; Undem and Nassenstein, 2009; Sato *et al.*, 2014), and activation of sensory nerves by ATP has been shown to be through P2X3 (Kwong *et al.*, 2008), it can be hypothesised that perhaps activation of TRPV4 induces ATP release and subsequent activation of P2X3. Furthermore, It has previously been shown in human epithelial cells that ATP release following TRPV4 activation is mediated through the Pannexin 1 ion channel (Seminario-Vidal *et al.*, 2011; Baxter *et al.*, 2014), and so the role of the Pannexin 1 ion channel will also be investigated.

5.1.2: Chapter Hypothesis:

Activation of TRPV4 on airway sensory nerves leads to the release of ATP and activation of P2X3.

5.1.3: Aims

- To confirm that P2X3 is expressed on vagal ganglia, and in addition that agonists are capable of activating airway sensory nerves. This will be carried out using $\alpha\beta$ -MethyleneATP ($\alpha\beta$ -MeATP), a more stable agonist at P2X1 and P2X3 receptors and investigating its effects on calcium movement and vagal nerve depolarization. Two structurally distinct antagonists will be used; TNP-ATP and AF-353, to confirm the role of the P2X3 receptor.
- Examine the effect of selective P2X3 antagonists against TRPV4 mediated activation of airway sensory nerves *in vitro* using calcium movement in guinea pig isolated airway ganglia and vagal nerve depolarization in isolated murine, guinea pig and human tissue.

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- Examine the role of Pannexin 1 in TRPV4 mediated depolarization using vagus from Pannexin 1 knockout mice.
- Examine the effect of a selective P2X3 antagonist AF-353 against TRPV4 mediated events *in vivo* using single airway nerve firing and reflex cough in the guinea pig.

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5.2: Methods

5.2.1: RT-PCR

Quantitative real time PCR assays for the P2X3 receptor were purchased from Applied Biosystems and validated using cDNA from tissue/organs from the guinea pig that expressed the target channel at high levels.

Whole nodose and jugular ganglia were harvested from male Dunkin Hartley guinea pigs. Expression levels of P2X3 were determined using the validated assays, with real time RT-PCR performed as described previously (Wong *et al.*, 2009) and in [Section 2.4](#).

5.2.2: Ganglia imaging

Male Dunkin Hartley guinea pigs were intranasally dosed with the retrograde tracer dye Dil to identify airway specific neurons, 14 days prior to dissection and enzymatic digestion of the nodose and jugular ganglia as described previously in [Section 2.5](#). Neurons were loaded into fluorodishes coated with laminin and allowed to adhere for 12 hours at 37°C 5%CO₂/95% O₂.

Following incubation, cells were loaded with the calcium specific dye Fura-2-AM for 40 minutes at 25°C in the dark, which was followed by a de-esterification step for 30 minutes at 25°C in the dark. Following a wash step with ECS, each fluorodish containing neurons was then placed in a full incubation chamber and mounted on a Widefield inverted microscope as described in [Section 2.5](#). Neurons were then identified using bright field illumination at x20 magnification, and airway specific cells could then be identified by imaging the cells at excitation and emission fluorescence wavelengths of approximately $\lambda=520-550\text{nm}$ and $\lambda=570$, which identifies the Dil labelled cells. Intracellular Ca²⁺ ([Ca²⁺]_i) responses were then investigated as previously described (Grace *et al.*, 2012; Dubuis *et al.*, 2013).

5.2.2.1: Agonist Responses

In order to determine if activation of P2X3 was able to cause a change in [Ca²⁺]_i signal in the nodose and jugular ganglia, the following experimental protocol was undertaken. Throughout the experiment, neurons were constantly superfused with ECS using an in house pressurised perfusion system that allows complete change of solution (600μL) in 3s. The neurons were exposed to a 50mM potassium solution (K₅₀) for 10s to ensure neuron viability, and allowed to return to baseline. αβ-MethyleneATP (αβ-MeATP; 10μM), a more stable agonist at P2X1 and P2X3 containing receptors was then added to the dish for 10 minutes, and changes in [Ca²⁺]_i recorded. The concentrations of agonist were chosen from previous publications (Weigand *et al.*, 2012).

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5.2.2.2: Antagonist responses

In order to determine if the P2X3 receptor played a role in TRPV4 mediated $[Ca^{2+}]_i$ signal, a pharmacological approach was used. Following identification of airway specific neurons, the P2X3 selective antagonist AF-353 (10 μ M (Weigand *et al.*, 2012)) was incubated with the ganglia cells for 10 minutes prior to agonist stimulation with GSK101 (30nM). A vehicle control (0.1% DMSO) was also carried out in cells from the same animal.

5.2.2.3: Analysis

Only responding cells (cells with a response $\geq$ 10% of the K_{50} response) and cells with a diameter of over 20 μ M were analysed as these were more likely to be sensory neurons (Hunter *et al.*, 2000). Intracellular calcium signal induced by each agonist was calculated using total area under the curve, (A.U.C.) which is the total change in intracellular calcium from resting level over time. This was then normalised to a percentage of the total change induced by K_{50} application, so that differences between individual cell responses can be accounted for. The data is presented as mean $\pm$ S.E.M., N indicates number of animals, and n indicates number of cells. Statistical analysis was carried out using an unpaired t test comparing cells pre incubated with vehicle prior to agonist treatment to cells preincubated with antagonist.

5.2.3: Isolated vagal nerve recordings

Vagal tissue was dissected from male Dunkin-Hartley guinea pigs, WT C57/Bl6 mice and $Px1^{-/-}$ mice and placed in a grease-gap recording chamber as previously described in [Section 2.6](#). Key experiments were repeated using human donor tissue to provide translational data.

5.2.3.1: Agonist concentration response curves

In order to determine if activation of P2X3 was able to depolarise the vagal nerve, the following experimental protocol was undertaken. Non-cumulative concentrations of the P2X3 agonist $\alpha\beta$ -MeATP (10-300 μ M) were applied to the vagal nerve trunk for two minutes followed by a wash period where the nerve was washed with Krebs solution until it returned to baseline. In addition, vehicle (0.1% dH₂O v/v) was tested on the nerve to ensure that it had no effect. Responses were recorded as mV depolarisation. Concentration ranges of each agonist were determined from published work (Weigand *et al.*, 2012). Responses were carried out in guinea pig tissue, and a submaximal concentration used to determine activation of human tissue.

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5.2.3.2: Antagonist responses

To ensure specificity for the P2X3 ion channel, two structurally distinct P2X3 antagonists were utilised; TNP-ATP (10 μ M) and AF-353 (10 μ M) against a submaximal concentration of $\alpha\beta$ -MeATP; (100 μ M). AF-353 is thought to be more selective for the P2X3 ion channel, whereas TNP-ATP blocks both P2X3 and the heteromeric P2X2/P2X3 channel. Concentrations of each antagonist were based on previous work (Weigand *et al.*, 2012) and published potency values (Virginio *et al.*, 1998; Gevers *et al.*, 2010). Once the antagonists were shown to inhibit P2X3 induced responses, the antagonists were then tested against TRPV4 (GSK101; 300nM) induced depolarisation in order to determine if P2X3 is involved in the mechanism of TRPV4 induced activation of airway sensory nerves. The antagonists were also profiled against capsaicin (1 μ M) and acrolein (300 μ M) induced depolarisation to determine selectivity.

A classical pharmacological approach was used; two reproducible responses to each agonist was established, following which each nerve was incubated with a single concentration of one of the antagonists for ten minutes. Following incubation, the nerve was restimulated with agonist in the presence of antagonist to determine inhibition. The nerve was then washed with Krebs until returned to baseline, and a final response to the agonist carried out to ensure nerve viability.

5.2.3.3: Analysis

Individual nerve depolarisations were measured in mV, and the chart recorder calibrated so that 1mm was equal to 0.01mV. Antagonist inhibitions were calculated by comparing the magnitude of depolarisation of the nerve following antagonist incubation to the mean magnitude of the two control agonist responses, to give a % inhibition. Statistical analysis was carried out using a two tailed t-test for matched pairs comparing the magnitude of depolarisation by the agonist with and without the presence of antagonist in the same piece of nerve. The data is presented as mean $\pm$ S.E.M., with statistical significance set at $P < 0.05$.

5.2.4: Genotyping of *Px1*^{-/-} Mice

The role of Pannexin 1 was investigated using Pannexin 1 knockout mice. Homozygous breeding pairs of mice genetically modified to disrupt the Pannexin 1 gene (*Px1*^{-/-}) were a kind gift from Dr. Vishva Dixit, (Genentech, US) and breeding colonies maintained on a C57Bl/6 background at Imperial College London. Regular genotyping was carried out to ensure continued deletion of the Pannexin 1 allele. This involved amplification of the DNA sequence of interest using PCR as previously described in [Section 2.3.1](#).

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DNA samples extracted from the tail tips of WT (C57Bl/6) and *Px1*^{-/-} mice were amplified by PCR using primers (Invitrogen) and visualised on an agarose gel by electrophoresis. Samples were heated to 94°C, following which 40 cycles of denaturing, annealing and extension were carried out, the exact duration and temperatures of which are outlined in **Table 5.1**. The resulting PCR products were run on 2% agarose gel (80V for 1 hour) in TBE buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd, Huntingdon, UK) alongside a 50-1000bp ladder (Hyperladder 50bp, Bioline Reagents, UK). The expected wild type product was 600bp and knock out 350bp.

Table 5.1: PCR Protocol for *Px1*^{-/-} mice

	PCR Conditions			
Pannexin	Denaturing 94°C 30 sec	Annealing 60°C 1min	Extension 72°C 1min	Product Size (Base Pairs) WT: 600 KO: 350

5.2.5: *In vivo* single fibre recordings

Male Dunkin Hartley guinea pigs (~350-400g) were anaesthetised with urethane (1.5g/kg i.p.) and paralysed with vecuronium bromide (0.1mg/kg i.v.) and ventilated. Heart rate, blood pressure and body temperature were constantly monitored for the duration of the experiment. Guinea pigs were then prepared as described in [Section 2.7.1](#) and as previously described (Adcock *et al.*, 2003). Briefly, both vagal nerves were cut at the cervical end but only the left vagal nerve used for recording. This 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. The nerve fibre was identified as originating from the family of SARs, RARs or chemosensitive C fibres using a set of criteria (Adcock *et al.*, 2003b) and as described in [Section 2.7.3](#). This included the pattern of spontaneous activity, response to hyperinflation and hyperdeflation, Adaptation Indices, response to capsaicin and citric acid and finally conduction velocity which was determined at the end of the experiment. For this set of experiments, only Aδ fibres were investigated.

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5.2.5.1: Agonist responses

The following experimental protocol was undertaken to determine the effect of $\alpha\beta$ Me-ATP on the firing of airway terminating vagal A δ -fibres. Following surgery, the animals were left to stabilise for 30 minutes, following which a control baseline recording was taken for 2 minutes. Vehicle (1% TWEEN80, 1% ETOH in saline v/v) was then aerosolised into the airways for up to 1 minute, and changes in fibre activity, intra-tracheal pressure and blood pressure were constantly monitored until they returned to baseline or a steady state achieved. 20 minutes later, citric acid (0.3M) was administered via aerosol into the airways and responses monitored. A further 20 minutes following return to baseline, $\alpha\beta$ Me-ATP (300 μ M; The concentration of $\alpha\beta$ Me-ATP which was based on concentrations shown to cause firing of airway fibres (Weigand *et al.*, 2012), was aerosolised into the airway for 1 minute and changes in fibre activity, intra-tracheal pressure and blood pressure closely monitored.

5.2.5.2: Antagonist responses

For antagonist experiments, only A δ -fibres were investigated. To investigate the antagonist activity of the P2X3 antagonist AF-353, two control responses to $\alpha\beta$ -MeATP: 300 μ M were carried out twenty minutes apart following which the antagonist or vehicle was administered i.p (AF-353: 30mg/kg in 10% Polyethylene Glycol 400 in 0.9% saline v/v, vehicle: 10% Polyethylene Glycol 400 in 0.9% saline v/v). One hour later, $\alpha\beta$ -MeATP was re-aerosolised into the airways and responses monitored, and twenty minutes later GSK1016790a (100ng/ml) was re-administered to the airways and responses monitored. A similar protocol was undertaken for the TRPV4 antagonist GSK2193874 where two control responses to the TRPV4 agonist GSK101 (100ng/ml) were carried out twenty minutes apart, following which antagonist or vehicle were administered i.p (GSK2193874: 300mg/kg in 6% Cavitron/2Hydroxypropyl- β -cyclodextrin in saline, Vehicle: 6% Cavitron/2Hydroxypropyl- β -cyclodextrin in saline v/v). One hour later, GSK1016790a (100ng/ml) was administered and responses monitored. Twenty minutes later, $\alpha\beta$ Me-ATP (300 μ M) was aerosolised into the airways and responses monitored. Changes in fibre activity, intratracheal pressure and blood pressure were continuously recorded throughout the experiment.

5.2.5.3: Analysis

The data is expressed as mean $\pm$ S.E.M. and agonist studies analysed using a paired students t-test comparing responses as absolute values after stimulus to baseline values immediately before the response. For antagonist responses in the same fibres a paired t-test was used comparing agonist responses following antagonist to control responses. For responses in

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different fibres, an unpaired t-test was used comparing responses in antagonist fibres to vehicle fibres.

5.2.6: Cough

Conscious, unrestrained guinea pigs were placed in individual transparent Perspex plethysmography chambers (Buxco, Wilmington NC, USA), as described in [Section 2.8](#). Cough was detected as previously described (Maher, *et al.*, 2009; Birrell *et al.*, 2009.). The cough response induced by GSK101 (30µg/ml) was tested against the specific P2X3 antagonist AF-353 (10mg/ml). Guinea pigs were injected i.p. with antagonist or vehicle (10% Polyethylene Glycol 400 in 0.9% saline), 1 hour prior to exposure to GSK101 (30µg/ml). GSK101 was aerosolised for 5 minutes, and the number of coughs counted for 10 minutes.

5.2.6.1: Analysis

In order to test inhibition of cough by the P2X3 antagonist, a one tailed Mann Whitney U test was used, which compared responses from the antagonist group to vehicle control. The data is presented as mean±S.E.M., with statistical significance set at $P < 0.05$.

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5.3: Results

5.3.1: P2X3 expression in nodose and jugular ganglia

Prior to functional characterisation, it was first confirmed that P2X3 was expressed in guinea pig ganglia, as these hold the cell bodies of the fibres which project to the lung. Guinea pig ganglia were utilised as they are the species of choice to measure sensory nerve driven responses *in vivo* and *in vitro*. Following validation of the assay using tissue with the most abundant expression, quantitative RT-PCR of whole nodose and jugular ganglia was carried out to investigate levels of P2X3 mRNA. Although P2X3 was determined to be expressed at the mRNA level in both ganglia, predominant expression was found in the nodose, the ganglia type previously shown to respond to the TRPV4 agonist GSK101 (**Figure 5.1**).

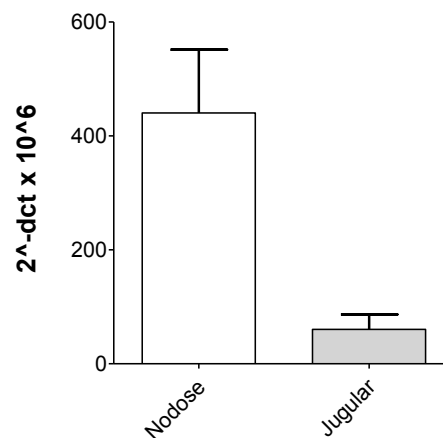


Figure 5.1: Expression of P2X3 in isolated guinea pig ganglia

P2X3 is expressed at the mRNA level in both nodose and jugular ganglia, with a higher level being present in the nodose. The data is expressed as mean ± S.E.M., n=8, and is normalised to the 18s control.

5.3.2: Effect of the P2X3 agonist $\alpha\beta$ -MeATP on depolarisation of isolated vagal nerves

In order to determine a functional effect of the P2X3 agonist $\alpha\beta$ -MeATP on airway sensory nerves, a concentration response curve to the agonist was carried out in guinea pig vagal tissue. The P2X3 agonist $\alpha\beta$ -MeATP (10-300 μ M) caused a concentration dependent increase in depolarization in the guinea pig vagal tissue (**Figure 5.2A**). $\alpha\beta$ -MeATP (100 μ M) caused a maximal depolarization of 0.17 ± 0.02 mV in guinea pig tissue, which was around 60-70% of the depolarization induced by the TRPV1 agonist capsaicin (1 μ M). $\alpha\beta$ -MeATP (100 μ M) also caused depolarization of isolated human tissue as indicated by the example trace (**Figure 5.2B**).

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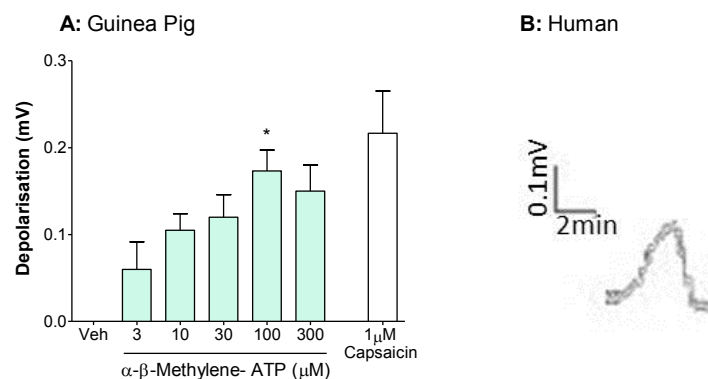


Figure 5.2: Effect of αβ-MeATP on depolarisation of isolated vagal tissue

*A: αβ-MeATP caused a concentration dependent depolarization of guinea pig isolated vagus nerve. B: 100 μM αβ-MeATP also caused depolarization of isolated human tissue. The data is shown as mean ± S.E.M. of n=4-6 for guinea pig vagal tissue. * indicates statistical significance at $p < 0.05$ using Kruskal Wallis test and Dunns post test comparing responses to vehicle.*

In order to determine if the depolarisation induced by αβ-MeATP was through the P2X3 ion channel, the ability of two structural distinct P2X3, P2X2/3 antagonists, TNP-ATP (10 μM) and AF-353 (10 μM) to inhibit αβ-MeATP induced depolarization was investigated. In guinea pigs, depolarization was inhibited 70.52±4.9% by TNP-ATP and 82.7±1.6% by AF-353 (**Figure 5.3A**). In human tissue responses were inhibited 90.9±9.1% by TNP-ATP and 88.9±11.11% by AF-353 (**Figure 5.3B**).

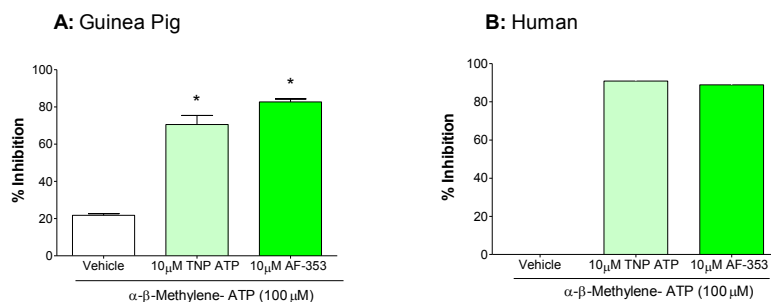


Figure 5.3: Effect of P2X3 antagonists on αβ-MeATP induced depolarisation

*A: The P2X2/3, P2X3 antagonist TNP-ATP (10 μM) and the P2X3 antagonist AF-353 (10 μM) significantly inhibited depolarisation induced by αβ-MeATP (100 μM) in guinea pig vagal nerves. B: A similar effect was seen in donor human tissue where both antagonists inhibited the depolarization induced by αβ-MeATP. Vehicle (Veh; 0.1% DMSO) had no effect on nerve activation induced by either agonist in any species tested. The data is presented as mean ± S.E.M. of n=4-6 observations, n=2 for human. Human tissue was obtained from 2 male and donors aged 38-57 with no known respiratory disease. * indicates statistical significance ($p < 0.05$), paired t-test comparing responses in the same piece of nerve with and without antagonist. Due to limited n numbers statistical analysis was not carried out on human tissue.*

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5.3.3: Effect of P2X3 agonists on $[Ca^{2+}]_i$ signal in nodose and jugular ganglia

A further functional effect of P2X3 in airway sensory nerves was investigated using $[Ca^{2+}]_i$ signal in guinea pig isolated nodose and jugular ganglia, where a change in $[Ca^{2+}]_i$ signal is indicative of activation of the ion channel. $\alpha\beta$ -MeATP (10 μ M), was shown to activate airway stained ganglia, with a predominant effect in the nodose ganglia (**Figure 5.4A**). This is a similar ganglia activation profile to that seen with TRPV4 agonists. Maximum Ca^{2+} signals were $56 \pm 21.8\%$ of K_{50} A.U.C in nodose ganglia, and $13.2 \pm 1.9\%$ in jugular ganglia. An example trace of change in $[Ca^{2+}]_i$ signal in the nodose ganglia following administration of $\alpha\beta$ -MeATP is shown in **Figure 5.4 B**.

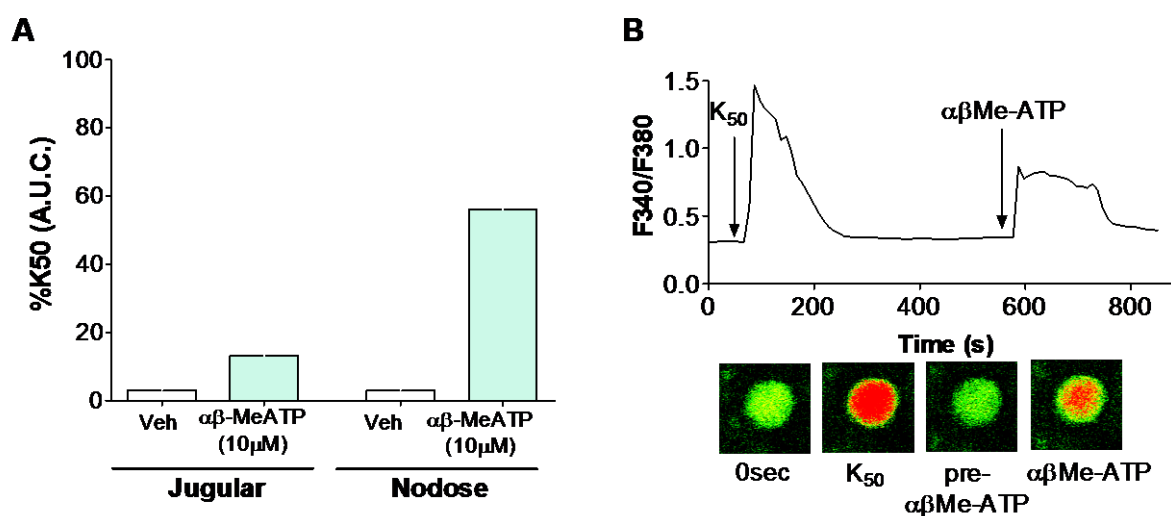


Figure 5.4: Effect of $\alpha\beta$ -MeATP on $[Ca^{2+}]_i$

A: $\alpha\beta$ -MeATP (10 μ M) caused an increase in $[Ca^{2+}]_i$ in both jugular and nodose ganglia as indicated by an increase in K_{50} A.U.C, however a larger response was found in the nodose ganglia. B: Indicates an example trace of calcium release over time. The trace shows calcium release over time, and corresponding images below. Increase in red indicates increase in $[Ca^{2+}]_i$. The data is presented as mean $\pm$ S.E.M. of N=2, n=2-4.

5.3.4: Effect of the P2X3 agonist $\alpha\beta$ -MeATP on single fibre firing

Finally the effect of $\alpha\beta$ -MeATP on action potential propagation in A δ -fibres was investigated. $\alpha\beta$ -MeATP (300 μ M) caused firing of all A δ -fibres investigated, but appeared not to cause bronchoconstriction, as there was no increase in tracheal pressure (**Figure 5.5**).

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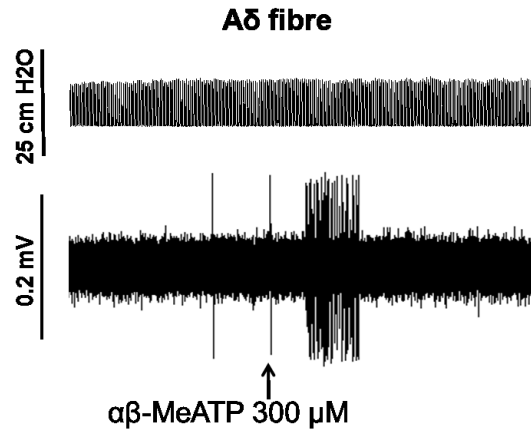


Figure 5.5: Example trace of an A δ fibre

Example trace of action potential firing induced by $\alpha\beta$ -MeATP (300 μ M) in an A δ fibre. $\alpha\beta$ -MeATP caused firing of the fibre (bottom panel), but had no effect on bronchoconstriction (top panel)

5.3.5: Effect of P2X3 antagonists on TRPV4 induced activation of airway sensory nerves *in vitro*

In order to determine if the P2X3 ion channel is involved in TRPV4 induced activation of airway sensory nerves, the 2 structurally distinct P2X3 antagonists TNP-ATP and AF-353 were investigated against both GSK101 (300nM) and -80mOsm induced depolarisation; which are exogenous and endogenous agonists of TRPV4 respectively.

Both TNP-ATP (10 μ M) and AF-353 (10 μ M) significantly inhibited depolarisation induced by both GSK101 (300nM) and -80mOsm. TNP-ATP (10 μ M) reduced the GSK101 induced depolarisation $94.1 \pm 5.9\%$ and -80mOsm by $73.1 \pm 10.3\%$ in the guinea pig. AF-353 (10 μ M) inhibited the GSK101 induced depolarisation $86.3 \pm 8\%$ and -80mOsm $85.6 \pm 7.2\%$ (**Figure 5.6A**), indicating that P2X3 is involved in the mechanism of TRPV4 induced depolarisation in the guinea pig.

A similar result was seen in donor human vagal tissue where depolarisation induced by GSK101 (300nM) was inhibited 100% by both antagonists. Depolarisation induced by -80mOsm was inhibited $40 \pm 20\%$ by TNP-ATP and $70 \pm 8.3\%$ by AF-353 (**Figure 5.6B**).

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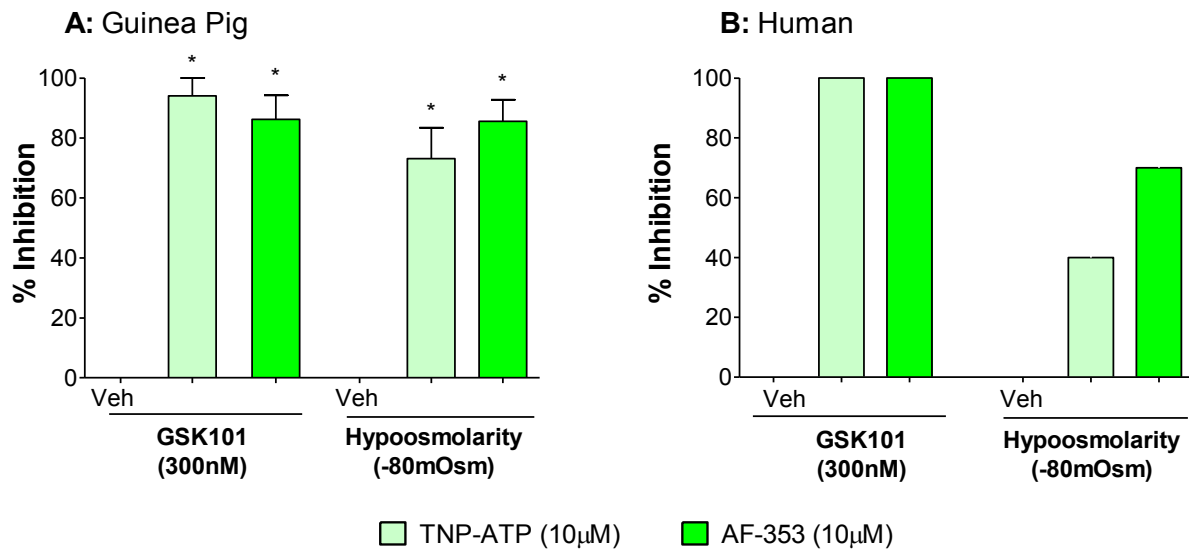


Figure 5.6: Effect of P2X3 antagonists on TRPV4 induced depolarisation

A: Guinea pig isolated vagus. Both TNP-ATP (10μM) and AF-353 (10μM) significantly inhibited depolarisation induced by the TRPV4 agonists GSK101 (300nM) and -80mOsm. B: Donor human isolated vagus. Both P2X3 antagonists also inhibited depolarisation induced by GSK101 (300nM) and -80mOsm in human vagal tissue. Vehicle (Veh; 0.1% DMSO) had no effect on nerve activation induced by either agonist in any species tested. The data is presented as mean ± S.E.M. of n=4-6 observations in guinea pig and n=2 in human tissue. Human tissue was obtained from 2 male and 1 female donor aged 38-57 with no known respiratory disease * indicates statistical significance (p<0.05), paired t-test comparing responses in the same piece of nerve with and without antagonist.

In order to ensure that this effect was unique to TRPV4 and the P2X3 antagonists were not just working as a global TRP inhibitors, AF-353 (10μM) was also tested against TRPV1 (Capsaicin: 1μM) and TRPA1 (Acrolein: 300μM) induced depolarisation. AF-353 (10μM) had no effect on either capsaicin or acrolein induced depolarisation, indicating that the inhibitory effect of the P2X3 antagonist is unique to TRPV4 (**Figure 5.7**).

5: The role of ATP and P2X3 in TRPV4 mediated activation of airway sensory nerves

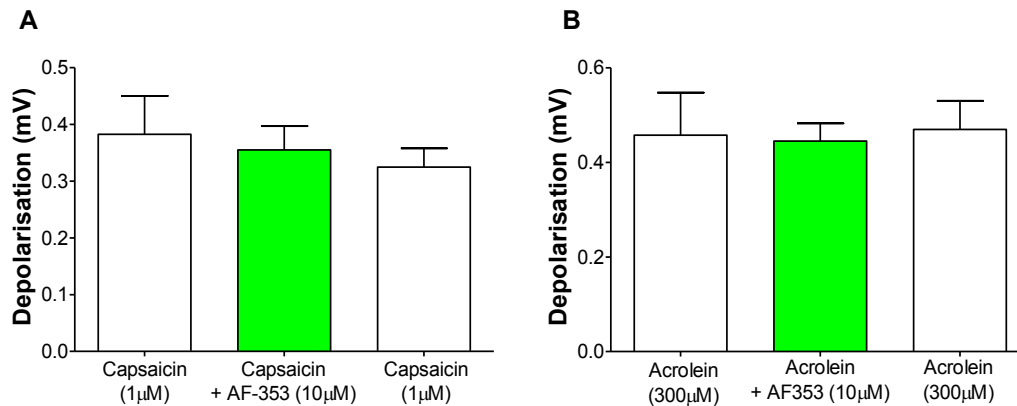


Figure 5.7: Effect of AF-353 on capsaicin and acrolein induced vagal nerve depolarisation

AF-353 (10µM) had no effect on either TRPV1 (capsaicin, A) or TRPA1 (acrolein, B) induced depolarisation in the guinea pig. The data is presented as mean ± S.E.M. of n=4 observations in guinea pig

In order to investigate if further functional effects induced by GSK101 (30nM) were also inhibited, AF-353 (10µM) was also tested in airway specific nodose neurons, where the P2X3 antagonist significantly inhibited $[Ca^{2+}]_i$ signal induced by GSK101 (30nM) (**Figure 5.8**).

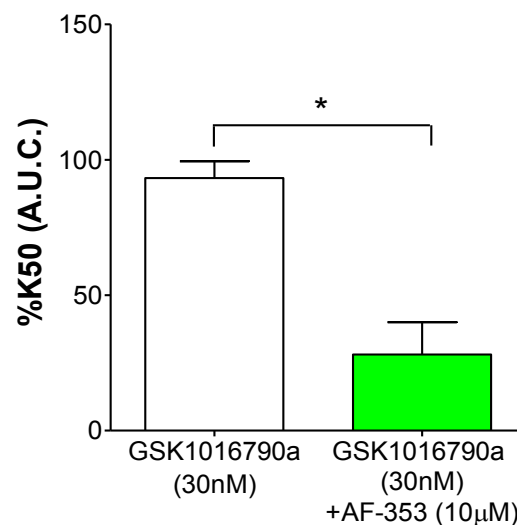


Figure 5.8: Effect of AF-353 on GSK101 induced $[Ca^{2+}]_i$

*$[Ca^{2+}]_i$ signal induced by GSK101 (30nM) was significantly inhibited following incubation with AF-353. N=4, n=5. * indicates statistical significance at $p < 0.05$ using unpaired t-test comparing cells incubated with vehicle prior to GSK101 (30nM) to cells incubated with AF-353 prior to GSK101 (30nM).*

5: The role of ATP and P2X3 in TRPV4 mediated activation of airway sensory nerves

5.3.6: Role of the Pannexin 1 ion channel

The body of data so far has confirmed the hypothesis that activation of TRPV4 leads to ATP release and activation of P2X3. Two recent studies have also suggested that TRPV4 induced release of ATP in human epithelial cells, and this release is associated with the Pannexin-1 channel (Seminario-Vidal *et al.*, 2011; Baxter *et al.*, 2014). To investigate if this signalling mechanism plays a role in TRPV4 mediated depolarisation, *Px1*^{-/-} mice were utilised. Mice are a useful model as the genetic template can be modified and knockouts can be utilised which allows the investigation of the importance of the Pannexin 1 receptor without pharmacological intervention.

Pairs of mice genetically modified to remove the Pannexin 1 gene were obtained and bred in house, and male homozygous knockout mice used for experiments. Regular genotyping was undertaken to ensure that the gene of interest was knocked out. An example gel for the *Px1*^{-/-} mice is shown in **Figure 5.9**. All mice were bred on a C57Bl/6 background, and therefore male C57Bl/6 mice were used as wild type controls.

In knockout mice, responses to the TRPV4 ligand GSK101 was virtually abolished (**Figure 5.10** $p < 0.05$). However responses to the P2X3 agonist $\alpha\beta$ -MeATP (100 μ M) alongside capsaicin (1 μ M) and acrolein (300 μ M) remained unchanged in both wild type and knockout tissue (**Figure 5.10**).

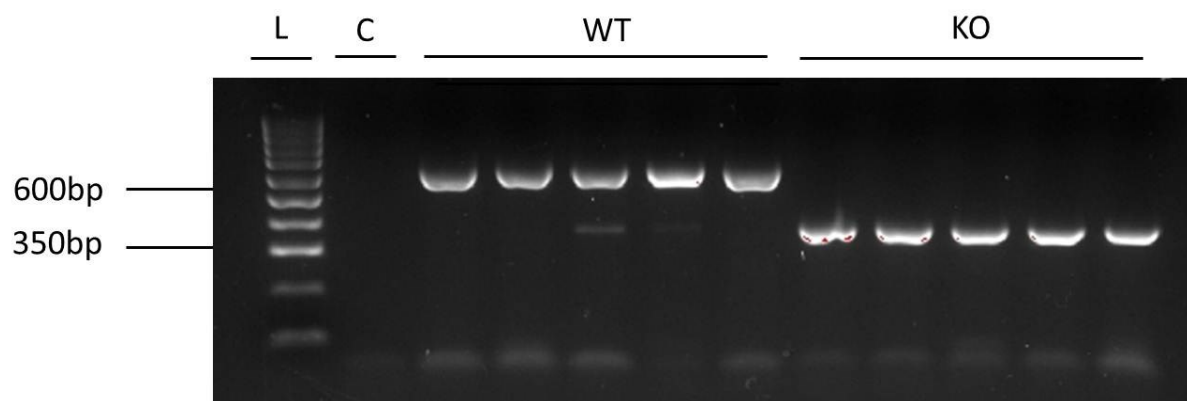


Figure 5.9: Example gel from *Px1*^{-/-} mice

RTPCR was used to confirm knockout of the Pannexin1 gene using DNA extracted from tail tips from mice used in Figure 5.10. Wild type mice produced a band at 600bp and KOs at 350bp. A 20-1000 ladder was used (L), and a water control (C). Wild type and knockout primers were run in the same reaction.

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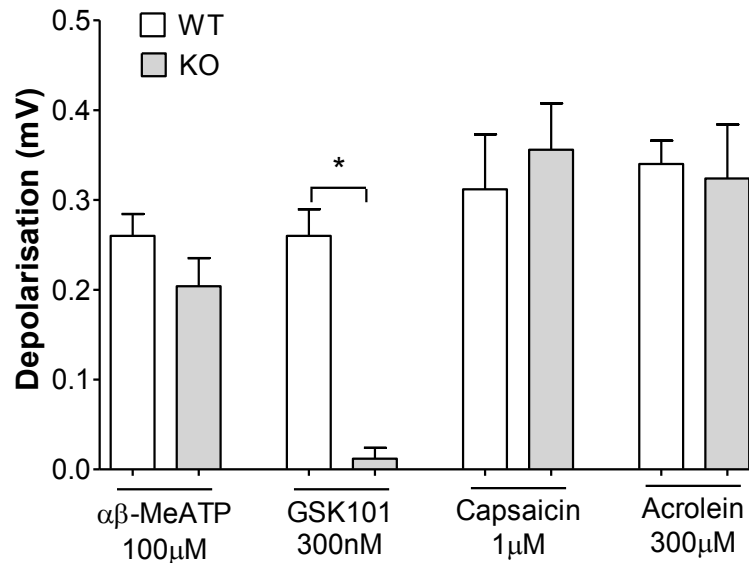


Figure 5.10: Effect of agonists in $Px1^{-/-}$ mice

Responses to GSK101 (300nM) were abolished in $Px1^{-/-}$ mice. Responses to $\alpha\beta$ -MeATP (100 μ M), capsaicin (1 μ M) and acrolein (300 μ M) remained unchanged. The data is presented as mean $\pm$ S.E.M.. of $n=4-6$. * indicates statistical significance ($p < 0.05$), unpaired t -test comparing responses in $Px1^{-/-}$ tissue with wild-type control.

5.3.7: Effect of P2X3 antagonists on TRPV4 induced activation of airway sensory nerves *in vivo*

As TRPV4 induced responses were inhibited *in vitro* following a P2X3 antagonist, it was next investigated if TRPV4 agonist responses were also inhibited *in vivo*. Initially the effect of the P2X3 and TRPV4 antagonists were investigated against both TRPV4 and P2X3 induced airway single fibre firing. In these set of experiments, only A δ -fibres were investigated. In all cases, citric acid activated the nerves under investigation and was used as an initial test for nerve fibre viability. To investigate the role of the P2X3 antagonist AF-353 on A δ firing induced by $\alpha\beta$ Me-ATP and GSK101 6 A δ -fibres were investigated with CVs ranging from 3.8-14.1 imp/s.

Two reproducible firing responses to $\alpha\beta$ -MeATP were obtained 20 minutes apart, following which either vehicle (10% Polyethylene Glycol 400 in dH₂O) or AF-353 (30mg/kg) were administered. Any changes in further responses to both $\alpha\beta$ -MeATP and GSK101 were then observed. In the vehicle treated animals, neither $\alpha\beta$ -MeATP nor GSK101 (100ng/ml) firing responses were inhibited (**Figure 5.11A,C**). However, following AF-353 (30mg/kg) administration, responses to both $\alpha\beta$ -MeATP and GSK101 were significantly inhibited, as indicated in the example trace (**Figure 5.11 B,D**). Inhibition of the $\alpha\beta$ -MeATP response was measured in the same fibre, whereas responses to GSK101 following antagonist were

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compared to those following vehicle administration. GSK101 caused a slow onset prolonged bronchoconstriction in all fibres investigated, which was unchanged following either vehicle or AF-353 administration (**Figure 5.11 E, F**).

In a separate set of experiments, the TRPV4 antagonist GSK2193874 (300mg/kg) was also investigated. A δ -fibres (N=6) were investigated with CVs ranging from 3.2-14.3 m/s. Two reproducible responses to GSK101 (100ng/ml) were carried out following which either vehicle or GSK2193874 (300mg/kg) were administered. Following vehicle administration firing to GSK101 was unchanged. $\alpha\beta$ -MeATP also caused firing of the fibres investigated. However, following administration of the GSK2193874 antagonist, firing induced by GSK101 (100ng/ml) was significantly inhibited. However firing induced by $\alpha\beta$ -MeATP remained unchanged from vehicle control indicating that antagonising TRPV4 does not inhibit P2X3 mediated responses (**Figure 5.12 A,B,C**). The TRPV4 antagonist also inhibited GSK101 induced bronchoconstriction (**Figure 5.12 D,E**).

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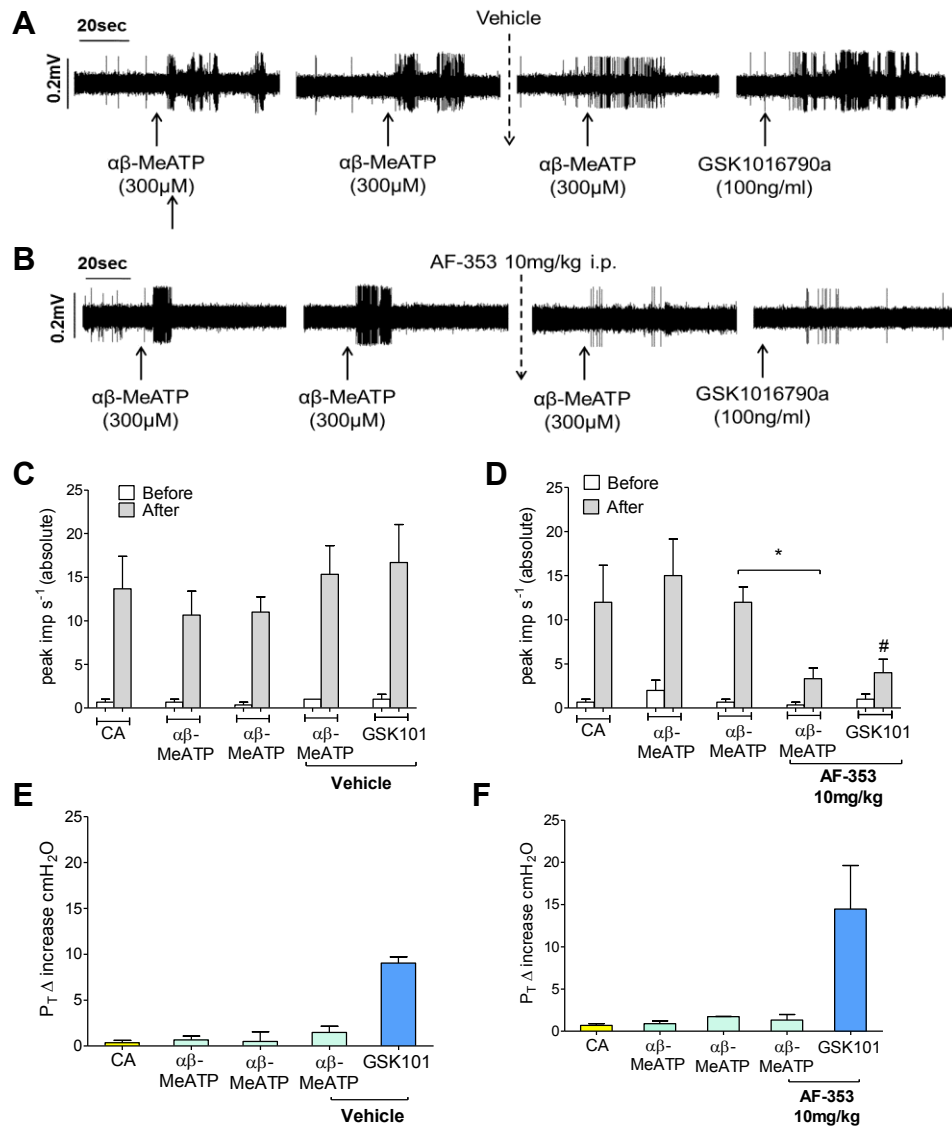


Figure 5.11: Effect of the P2X3 antagonist AF-353 on P2X3 and TRPV4 induced airway A δ fibre firing and bronchoconstriction

A: Example trace from a vehicle treated animal. Two reproducible responses to $\alpha\beta$ -MeATP (300 μ M) were carried out following which vehicle was administered i.p. The response to $\alpha\beta$ -MeATP (300 μ M) or GSK101 (100ng/ml) was unchanged. Graphical data in C. **B:** Example trace from an animal treated with AF-353 (30mg/kg). Two reproducible responses to $\alpha\beta$ -MeATP caused firing of the A δ -fibres investigated, AF-353 administration inhibited $\alpha\beta$ -MeATP induced firing. GSK101 induced firing was also significantly inhibited compared to the vehicle control. Graphically represented in D.

E. and **F** indicate that neither CA nor $\alpha\beta$ -MeATP (300 μ M) have any effect on bronchoconstriction, and the bronchoconstriction induced by GSK101 remains unchanged following vehicle or AF-353 administration. Data represents mean $\pm$ S.E.M of n=3 for vehicle and n=3 for antagonist observations.

* indicates statistical significance ($p < 0.05$), paired t test comparing responses before and after aerosol administration of agonist or antagonist. # indicates statistical significance ($p < 0.05$) using unpaired t test comparing responses in antagonist treated fibres to vehicle treated fibres.

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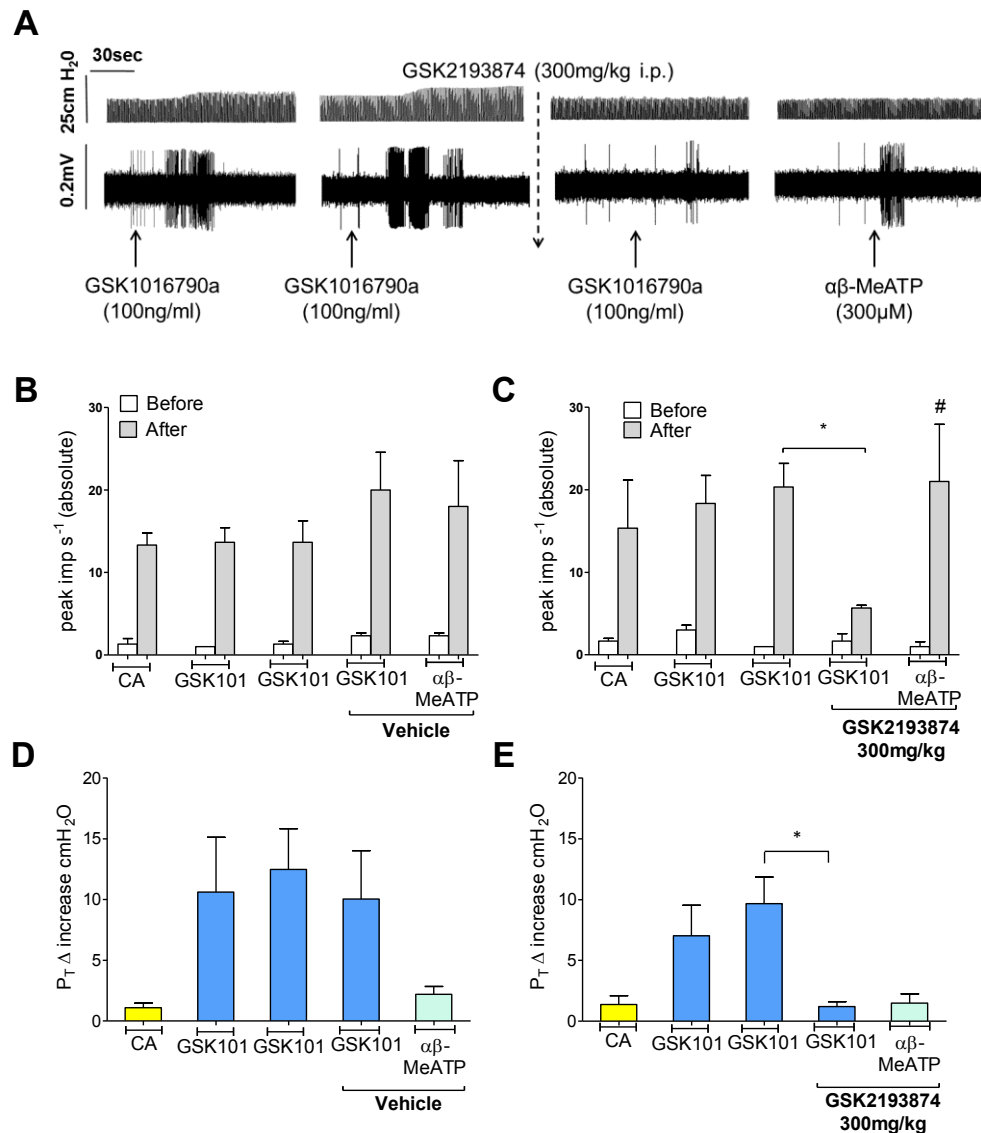


Figure 5.12: Effect of the TRPV4 antagonist GSK2193874 on TRPV4 and P2X3 induced airway Aδ fibre firing

A: Example trace from an antagonist treated animal. Two reproducible responses to GSK101 (100ng/ml) were carried out following which GSK2193874 (300mg/kg) was administered i.p. The response to GSK101 (100ng/ml) was inhibited, however αβ-MeATP (300μM) response was unchanged. This data is expressed graphically in C. **B:** Data from vehicle treated animals. Responses to GSK101 (100ng/ml) or αβ-MeATP (300μM) were unchanged following vehicle administration. **D** and **E** indicate that the TRPV4 antagonist significantly inhibited the bronchoconstriction induced by GSK101. Data represents mean±S.E.M of n=3 for vehicle and n=3 for antagonist observations. * indicates statistical significance ($p < 0.05$), paired t test comparing responses before and after aerosol administration of agonist or antagonist. # indicates statistical significance ($p < 0.05$) using unpaired t test comparing responses in antagonist treated fibres to vehicle treated fibres.

5: The role of ATP and P2X3 in TRPV4 mediated activation of airway sensory nerves

Finally the ability of the P2X3 antagonist AF-353 to inhibit GSK101 induced reflex cough in the conscious guinea pig was investigated. Following i.p. administration of AF-353 (30mg/kg), GSK101 (30 μ g/ml) induced cough was inhibited to a similar level as seen with the TRPV4 antagonist, indicating that inhibiting P2X3 also inhibits GSK101 induced reflex events (**Figure 5.13**).

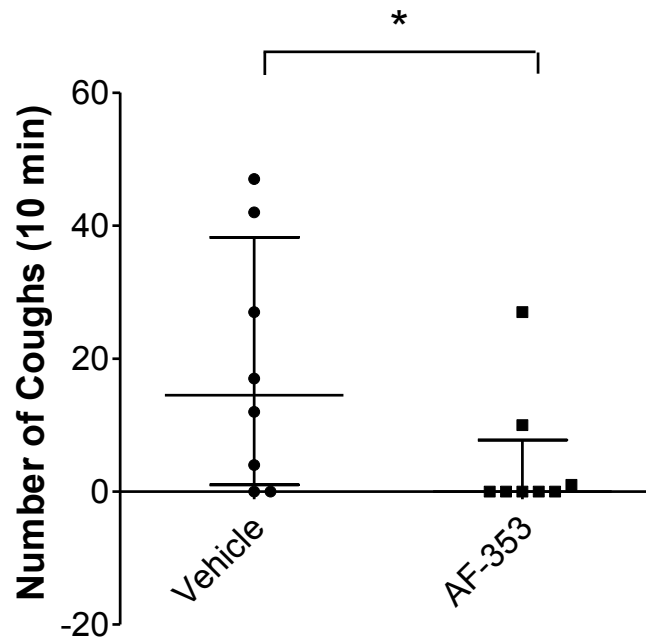


Figure 5.13: Effect of AF-353 on TRPV4 induced cough

AF-353 inhibited cough induced by the TRPV4 agonist GSK101 (30 μ g/ml) compared to vehicle control. Data is displayed as median $\pm$ interquartile range of 8 observations * indicates statistical significance at $p < 0.05$ using a one tailed Mann Whitney U test.

5: The role of ATP and P2X3 in TRPV4 mediated activation of airway sensory nerves

5.4: Discussion

The aim of this chapter was to determine if P2X3 and ATP are involved in the mechanism of TRPV4 activation. In **Chapters 3 and 4**, TRPV4 agonists were shown to activate nodose derived A δ -fibres in the guinea pig, and both calcium signalling and fibre firing induced by TRPV4 demonstrated a short delay prior to activation indicating the involvement of a secondary mediator. A possible candidate for this secondary mediator is ATP, as previous work in the bladder has suggested that TRPV4 can lead to ATP release and subsequent activation of P2X3 on bladder afferents (Mochizuki *et al.*, 2009; Aizawa *et al.*, 2012). Furthermore, a study in human epithelial cells has indicated that cigarette smoke induced ATP release is mediated through the TRPV4 ion channel (Baxter *et al.*, 2014). P2X3 has been shown to be expressed in both guinea pig and human nodose ganglia (Kwong *et al.*, 2008; Sato *et al.*, 2014). P2X3 is an ionotropic purinergic receptor which is primarily expressed in sensory nerves (Burnstock and Verkhratsky, 2010) and $\alpha\beta$ Me-ATP, a more stable agonist at P2X1 and P2X3 containing receptors, is a potent agonist. From this, it can be hypothesised that activation of TRPV4 causes a calcium influx, which evokes ATP release, which in turn activates the P2X3 ion channels present on airway sensory nerves. This is of particular interest as recently a P2X3 antagonist AF-219, has been shown to significantly inhibit daytime objective cough frequency in patients with idiopathic chronic cough (Abdulqawi *et al.*, 2014). Furthermore, ATP levels have been shown to be elevated in patients with asthma and COPD (Mohsenin and Blackburn, 2006; Polosa and Holgate, 2006; Idzko *et al.*, 2007; Lommatzsch *et al.*, 2010; Mortaz *et al.*, 2010), where airway sensory nerve responses are thought to be enhanced. In addition, aerosolised ATP caused cough, dyspnea and tightness in asthmatics (Basoglu *et al.*, 2005), smokers and patients with COPD (Basoglu *et al.*, 2015).

Extracellular ATP can activate two subsets of the cell surface purinergic P2 receptors, the ionotropic P2X receptors and the G protein-coupled P2Y receptors (Burnstock and Kennedy, 1985). The ion channel P2X3 is a member of the ionotropic P2X family which are widely expressed in neuronal, epithelial, muscular and immune cells (Burnstock and Knight, 2004) and function as either homo or heterotrimers formed of protein subunits encoded by the genetically distinct P2X1-7 subtypes (North and Surprenant, 2000; Gever *et al.*, 2006). The expression of P2X3 however, is primarily limited to sensory neurons (Chen *et al.*, 1995; Burnstock, 2008) and is formed by homo/heterotrimeric assembly of P2X2 and P2X3 receptors (Burnstock and Verkhratsky, 2010). P2X3 is expressed in a large proportion of C- and A δ -fibres (Kwong *et al.*, 2008; Undem and Nassenstein, 2009), which are known to be important for the detection of noxious stimuli in the lung. P2X3 has been shown to be involved in the activation of these fibres, which are critical to cough initiation and sensitisation (Undem and Nassenstein, 2009). In addition, extracellular ATP has been shown to cause firing of both

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canine and rodent A δ and C fibres (Pelleg and Hurt, 1996; Kwong *et al.*, 2008), which was determined to be blocked by the P2X3/ P2X2/3 antagonist A-317491 (Kwong *et al.*, 2008). Alongside causing direct activation of fibres, ATP has also been shown to enhance citric acid induced cough in guinea pigs which was inhibited with pretreatment with the P2X3/P2X2/3 antagonist TNP-ATP. ATP had no effect however on capsaicin induced cough (Brouns *et al.*, 2000).

Before determining if P2X3 and ATP play a role in TRPV4 mediated activation of airway sensory nerves, it was firstly determined that the P2X3 agonist $\alpha\beta$ -MeATP was able to activate airway sensory nerves. Expression of P2X3 was confirmed using RTPCR of the jugular and nodose ganglia, with a higher expression level found in the nodose, similar to the TRPV4 expression profile. A concentration response to the P2X3 agonist $\alpha\beta$ -MeATP was then carried out in the guinea pig isolated vagus ganglia, where $\alpha\beta$ -MeATP caused a concentration dependent increase in depolarization of the guinea pig vagus nerve, and a submaximal concentration (100 μ M) also caused depolarization of donor human vagal nerves. This depolarization was shown to be specific to the P2X3 ion channel as two structurally distinct antagonists AF-353 (10 μ M) and TNP-ATP (10 μ M) significantly inhibited depolarization induced by the P2X3 agonist in both guinea pig and human tissue. When tested on airway specific ganglia, $\alpha\beta$ -MeATP was also shown to cause an increase in $[Ca^{2+}]_i$, and although this was seen to a small degree in the jugular ganglia, the predominant effect was seen in the nodose. Finally, $\alpha\beta$ -MeATP was shown to cause firing of airway terminating A δ -fibres *in vivo*. Taken together, this indicates that the P2X3 agonist $\alpha\beta$ -MeATP is capable of activating airway sensory nerves both *in vitro* and *in vivo*, and the activation profile is very similar to that seen by the TRPV4 agonist GSK101 as shown in **Chapter 3**.

To determine the role of P2X3 in TRPV4 mediated responses, the P2X3 antagonists AF-353 and TNP-ATP were then tested against TRPV4 mediated activation of airway sensory nerves. In isolated guinea pig vagal tissue, P2X3 antagonists significantly inhibited depolarisation induced by both the TRPV4 antagonist GSK101, along with hypoosmolar solution, to a similar extent as the selective TRPV4 antagonists shown in previous chapters. This effect was mirrored in human tissue indicating that the results may translate to the clinic. To determine if this inhibition was a global effect on TRP channels, the antagonists were also profiled against TRPV1 and TRPA1 mediated responses where they had no effect, indicating that the inhibition is likely to be specific to TRPV4. The effect of AF-353 was also investigated against $[Ca^{2+}]_i$ in nodose ganglia, where the antagonist significantly inhibited the majority of calcium signal induced by the TRPV4 agonist GSK101 indicating that TRPV4 sensory nerve driven responses were indeed inhibited by P2X3 antagonists *in vitro*.

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Several studies in airway epithelial cells have suggested that activation of TRPV4 can lead to ATP release via the Pannexin-1 pore. Pannexins are a family of plasma membrane proteins and Pannexin 1 primarily functions as an ATP release channel (Bao *et al.*, 2004). Seminario-Vidal *et al* indicated that hypotonicity induced ATP release was inhibited following antagonism of Pannexin 1 and also in Pannexin 1 (*Px1*^{-/-}) knockout mice, and Baxter *et al* have shown that ATP release induced by a TRPV4 agonist was reduced following use of a Pannexin 1 antagonist (Seminario-Vidal *et al.*, 2011; Baxter *et al.*, 2014). Furthermore, it was shown that ATP release through Pannexin 1 was downstream of RhoA activation and rho kinase dependent myosin light chain phosphorylation (Seminario-Vidal *et al.*, 2011) indicating that TRPV4 activation may lead to RhoA activation. In this study, TRPV4 induced depolarisation of isolated vagal nerves was abolished in *Px1*^{-/-} knockout mice, whereas responses to TRPV1 (capsaicin), TRPA1 (acrolein) and P2X3 ($\alpha\beta$ -MeATP) ligands remained unchanged. This data indicates that Pannexin 1 has a critical involvement in ATP release induced following activation of the TRPV4 ion channel in the mouse vagus.

The P2X3 antagonist AF-353 was then taken forward to test against TRPV4 mediated effects on single airway fibres *in vivo*. TNP-ATP was not taken forward as the compound has low metabolic stability which is not ideal for *in vivo* studies (Jarvis *et al.*, 2001; Honore *et al.*, 2002; Ueno *et al.*, 2003). Firstly, AF-353 was tested against both $\alpha\beta$ -MeATP and GSK101 induced firing in guinea pig A δ -fibres. Responses to $\alpha\beta$ -MeATP were compared in the same animal after either antagonist or vehicle administration, whereas GSK101 induced responses were compared between antagonist treated and vehicle treated animals. Following antagonist administration, firing induced by $\alpha\beta$ -MeATP (300 μ M) were significantly inhibited in the A δ -fibres investigated. Furthermore, firing responses to the TRPV4 agonist GSK101 (100ng/ml) were significantly inhibited in the antagonist treated animals when compared to those that were dosed with vehicle. In addition, $\alpha\beta$ -MeATP had no effect on bronchoconstriction in any of the animals investigated, whereas GSK101 caused a slow onset, prolonged bronchoconstriction in all fibres investigated similar to that shown previously. This indicates that the P2X3 antagonist is also capable of inhibiting TRPV4 mediated A δ -fibre firing *in vivo*. However, interestingly the antagonist had no effect on GSK101 induced tracheal pressure, which indicates that the underlying mechanism involved in TRPV4 mediated bronchoconstriction is likely to be independent of a direct effect on P2X3 on the airway smooth muscle. P2X3 is predominantly expressed on airway sensory nerves further indicating that P2X3 is unlikely to play a direct role in TRPV4 mediated bronchoconstriction. However, as both of the vagal nerves were cut, this does not rule out a role for P2X3 in reflex bronchoconstriction.

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As a control measure, the TRPV4 antagonist GSK2193874 was also tested against GSK101 and P2X3 induced fibre firing in guinea pig A δ -fibres. In this case, responses to GSK101 were compared in the same animal after either antagonist or vehicle administration, whereas $\alpha\beta$ -MeATP induced responses were compared between antagonist treated and vehicle treated animals. GSK2193874 significantly inhibited TRPV4, but not $\alpha\beta$ -MeATP mediated firing in the fibres, indicating that activation of P2X3 by ATP is downstream of activation of TRPV4. GSK2193874 also inhibited GSK101 induced bronchoconstriction in all fibres investigated.

Finally the effect of AF-353 was assessed against GSK101 (30 μ g/ml) induced cough in the conscious guinea pig. Following i.p. administration of AF-353, GSK101 induced cough was significantly inhibited to a similar extent as with the TRPV4 antagonist GSK2193874 shown in **Chapter 3**. This indicates that AF-353 is capable of inhibiting TRPV4 mediated reflex events in the whole animal.

In summary, this body of data would suggest that the chapter hypothesis 'Activation of TRPV4 on airway sensory nerves leads to the release of ATP and activation of P2X3' can be accepted. Activation of airway sensory nerves both *in vitro* and *in vivo* was inhibited by two structurally distinct P2X3 antagonists, AF-353 and TNP-ATP, which had no effect on activation induced by other TRP agonists. The data would suggest that activation of TRPV4 on nodose derived A δ -fibres induces a small, local increase in $[Ca^{2+}]_i$ which in turn possibly could lead to RhoA activation and myosin light chain phosphorylation which then leads to the release of the neurotransmitter ATP through the Pannexin 1 ion channel. This then activates the ionotropic P2X3 channel, which is also present on A δ -fibres. It is this activation of P2X3 that then leads to the subsequent activation of vagal reflex events such as cough. This has previously been suggested to occur in bladder afferents where hypotonicity or stretch cause ATP release and subsequent activation of P2X3, and GSK101 evoked currents are blocked by a P2X3 antagonist (Birder *et al.*, 2007; Mochizuki *et al.*, 2009; Aizawa *et al.*, 2012).

Chapter 6:

Summary and Future Studies

6. Summary and Future Studies

6.1: Summary of thesis

A persistent cough is currently the most common reason for visiting a doctor in the UK (Schappert and Burt, 2006; Schappert and Rechtsteiner, 2011). Normally a defensive reflex which protects the lungs and airways from harmful particles, in certain inflammatory diseases such as asthma and COPD, the cough response can become augmented leading to excessive coughing. Chronic cough is a debilitating condition which can affect up to 40% of the population at any one time (Cullinan, 1992; Janson *et al.*, 2001; Morice *et al.*, 2001). Despite the prevalence there are currently no safe and effective therapies for treatment. Patients with asthma and COPD have shown increased cough sensitivity to inhaled tussive stimuli, suggesting that targeting the peripheral arm of the reflex could be effective (Key *et al.*, 2010). Therefore treatments which target ion channel and receptors present on the peripheral arm of the cough reflex could provide novel therapeutics.

Cough is a reflex carried out by airway sensory nerve fibres housed within the vagus nerve. Ion channels present on the vagus nerve have been shown to be activated by a wide range of stimuli which are found in the airways of patients with inflammatory airway diseases including an altered airway pH, ROS, changes in temperature, arachidonic acid derivatives and an altered airway osmolality. Two member of the TRP family of ion channels; TRPV1 and TRPA1 have been well documented in the activation of airway sensory nerves and the cough response (Birrell *et al.*, 2009; André *et al.*, 2008; Grace *et al.*, 2012; Lieu *et al.*, 2012), however the role of other TRP channels, in particular TRPV4, remains relatively unexplored.

TRPV4 is a calcium permeable ion channel which has been shown to be expressed in a number of cells within the airways including human airway smooth muscle cells, lung tissue, and a human bronchial epithelial cell line (Jia *et al.*, 2004; Alvarez *et al.*, 2006; Dietrich *et al.*, 2006; Yang, 2006; Fernández-Fernández *et al.*, 2008). TRPV4 has also been shown to be expressed at the mRNA level in pulmonary sensory neurons (Ni *et al.*, 2006). A role for TRPV4 in the airways has been eluded to as TRPV4 has been shown to cause contraction of human airway smooth muscle (Jia *et al.*, 2004; McAlexander *et al.*, 2014), and 7 SNPs of the TRPV4 gene are associated with developing COPD (Zhu *et al.*, 2009). The aim of this thesis was to determine a role for TRPV4 in airway sensory nerves and to investigate a possible mechanism of action.

Initially, TRPV4 mRNA expression was confirmed in guinea pig ganglia, where interestingly it was found to be predominantly expressed in the nodose. As there is currently a lack of selective TRPV4 antibodies and therefore protein levels could not be measured, functional expression was determined using calcium imaging of airway specific guinea pig ganglia. The

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selective TRPV4 agonist GSK101 caused a concentration dependent increase in intracellular calcium signal in the nodose ganglia, which was noticeably slow in onset. The agonist had no effect on calcium signal from the jugular ganglia, which is activated by TRPV1 and TRPA1 agonists, indicating an initial difference with the activation profile of TRPV4. In general it is thought that the jugular ganglia provide the more chemosensitive C fibres, and the nodose ganglia the more mechanosensitive A δ fibres (Canning *et al.*, 2006), suggesting that TRPV4 may play a role in activation of A δ fibres.

The next step was to pharmacologically characterise two structurally different selective TRPV4 agonists; GSK101 and 4 α PDD, in the isolated vagal preparation. Guinea pig and murine tissue were utilised, as guinea pigs have a cough reflex similar to man and although mice do not cough, the afferent arm of the cough reflex acts in a similar manner (Maher and Belvisi, 2010). The use of mice also allows the use of transgenic animals which allows the investigation of the importance of a particular receptor, in this case TRPV4, without pharmacological intervention. Both GSK101 and 4 α PDD were shown to cause a concentration dependent increase in depolarisation in both species, which was blocked by two structurally different TRPV4 antagonists GSK2193874 (10 μ M) and HC067047 (10 μ M), and abolished in *Trpv4*^{-/-} mice. Specificity of the ligands for the TRPV4 receptor was determined as neither a TRPA1 (HC030031: 10 μ M) nor a TRPV1 (JNJ17203212 100 μ M) antagonist had any effect on TRPV4 responses, and the TRPV4 antagonists had no effect on TRPV1 (Capsaicin 1 μ M) or TRPA1 (Acrolein 300 μ M) induced responses in the guinea pig or mouse.

Importantly, key experiments were repeated in human tissue to ensure that the results seen were translatable to the clinic. Donor human vagus was obtained, and both TRPV4 agonists were shown to cause depolarisation which was blocked by the TRPV4 antagonists, indicating that TRPV4 is also likely to play a role in human vagal nerve responses.

As the vagus nerve houses a variety of fibre types, it was not able to distinguish the fibre type responsible for TRPV4 mediated activation of the vagus nerve *in vitro*. Therefore the more potent of the 2 ligands; GSK101, was taken forward into the *in vivo* guinea pig isolated single fibre technique (Adcock, 2009) where its effect on fibre firing was investigated. GSK101 was shown to produce a slow onset, significant and sustained firing of the mechanosensitive A δ -fibres, but had no effect on the chemosensitive C-fibres. This was shown to be selective to TRPV4 as the selective TRPV4 antagonist GSK2193874 (300mg/kg) inhibited the response. This is concurrent with the results from the guinea pig ganglia, which indicated that GSK101 caused an increase in calcium signal in the nodose, but not the jugular ganglia. This is of particular interest as both TRPV1 and TRPA1 ligands have previously been shown to mostly activate C-fibres (and A δ nociceptors as TRPV1 is expressed), and not A δ -fibres, (Canning,

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2006), suggesting that the TRPV4 channel plays a role in the cough reflex via a novel mechanism of action. GSK101 was then taken forward into a conscious animal in a cough provocation study, where GSK101 was shown to cause cough in the conscious animal, which was blocked following i.p. administration of either HC067047 (100mg/kg) or GSK2193874 (300mg/kg), indicating that TRPV4 agonists are capable of causing a tussive response in the conscious animal.

This chapter had outlined a role for TRPV4 in both airway sensory nerves and cough through the activation of nodose derived A δ mechanosensitive fibres, a different and novel mechanism to that seen by activation of TRPV1 and TRPA1 channels, which do not ordinarily activate these fibres in healthy airways. The activation of mechanosensitive fibres may be due to the reported role of TRPV4 in osmoregulation, where osmotic solutions can provide a mechanical stimulus through cell swelling or cell shrinkage. TRPV4 was initially characterised as a sensor of osmotic stress (Strotmann *et al.*, 2000; Liedtke and Friedman, 2003; Suzuki *et al.*, 2003). Therefore, for the next chapter, the role of TRPV4 in osmolarity induced activation of airway sensory nerves was investigated.

Solutions of both hyper- and hypoosmolarity have been shown to cause activation of airway sensory nerves and reflexes such as cough in both animals and man (Fox *et al.*, 2005.; Eschenbacher *et al.*, 1984; Fuller and Collier, 1984; Laloo *et al.*, 1995), however the mechanisms behind activation have not been elucidated. Furthermore, airway disease patients including those with chronic cough and asthma have been shown to have a heightened tussive response to osmolar stimuli (Koskela *et al.*, 2008), and airway osmolarity has been shown to be altered in disease (Joris *et al.*, 1993). Hypoosmotic solutions have been shown to activate TRPV4 in many cell types including airway smooth muscle (Alessandri-Haber *et al.*, 2003; Jia *et al.*, 2004), however the role of TRPV4 in osmotic activation of airway sensory nerves is unknown.

Initially, solutions of increased (prepared using increasing concentrations of sucrose) and decreased (prepared by salt reduction and compensation with sucrose) osmolarity were prepared and characterised on isolated guinea pig, murine and donor human vagal nerves, where they were shown to cause concentration dependent depolarisation in all species. To determine the role of TRPV4, along with TRPA1 and TRPV1 in this activation, selective antagonists for each of the channels were used against depolarisation induced by both hyper and hypoosmolarity. It was determined that the TRPV4 antagonist (HC067047: 10 μ M) inhibited the majority of depolarisation induced by hypoosmolar solution, with the remainder of the depolarisation inhibited by a TRPV1 antagonist (JNJ17203212: 100 μ M) in guinea pig and human tissue. Vagal nerve responses from knockout mice showed a similar profile, where

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responses to hypoosmotic solution were significantly inhibited in both *Trpv1*^{-/-} and *Trpv4*^{-/-} mice. However, in contrast, TRPV4 was shown to play less of a role in hyperosmotic induced depolarisation; where, using knockout mice and selective antagonists in guinea pig and human tissue, the majority of activity was shown to be mediated through the TRPV1 ion channel.

As activation of sensory nerves by hypoosmotic solution *in vitro* was shown to be mediated predominantly through the TRPV4 ion channel in all species investigated, we investigated the effect on single airway nerve fibre firing in the guinea pig *in vivo*. A concentration range of solutions (from 0 to -100mOsm) were shown to activate both A δ - (similarly to the synthetic TRPV4 agonist GSK101), but only -100mOsm was able to significantly increase firing in C-fibres, indicating that A δ -fibres were more sensitive to smaller changes in osmolarity.

This chapter suggested a likely role for TRPV4 in hypoosmotic induced activation of airway sensory nerves, however less of a role in hyperosmotic induced activation. This is in agreement with previous data which have shown that hypoosmotic solutions cause activation of the TRPV4 ion channel *in vitro* (Alessandri-Haber *et al.*, 2003; Jia *et al.*, 2004).

The final aim of this thesis was to investigate a possible mechanism of TRPV4 activation of airway sensory nerves. Both fibre firing and calcium release observed in **Chapter 3** following administration of a TRPV4 agonist was slow in onset, potentially indicating the release of a secondary mediator. It was hypothesised that this secondary mediator could be ATP, as previous work in bladder sensory nerves has indicated that activation of TRPV4 can lead to ATP release and subsequent activation of P2X3 (Mochizuki *et al.*, 2009; Aizawa *et al.*, 2012). Furthermore, work in human epithelial cells has shown that cigarette smoke induced ATP release is inhibited following use of a TRPV4 antagonist (Baxter *et al.*, 2014).

P2X3 is an ionotropic purinoceptor predominantly expressed on sensory nerves (Chen *et al.*, 1995; Burnstock, 2008), and the stable derivative; $\alpha\beta$ Me-ATP, is a potent agonist. P2X3 can function as either a homotrimer consisting of three subunits of P2X3, or as a P2X2/3 heterotrimer consisting of subunits of P2X2 along with P2X3 (Burnstock and Verkhratsky, 2010). It was shown that P2X3 was present on predominantly the nodose ganglia in the guinea pig, similar to the TRPV4 channel, and the selective ligand $\alpha\beta$ Me-ATP caused a concentration dependent depolarisation in guinea pig vagus, which was blocked by two structurally different P2X3 antagonists AF-353 and TNP-ATP, induced calcium flux in airway specific nodose ganglia and induced firing of A δ -fibres.

In order to determine if P2X3 and ATP played a role in TRPV4 mediated activation of airway sensory nerves, the two structurally different P2X3 antagonists were tested against both

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GSK101 and hypoosmolar induced depolarisation, where responses were inhibited in guinea pig and human vagus. The antagonists however had no effect on responses induced by TRPV1 and TRPA1 agonists indicating that the responses were selective to TRPV4. AF-353 was also shown to inhibit TRPV4 induced calcium flux in airway stained guinea pig nodose ganglia cells, indicating that this response also occurs in airway specific sensory nerves.

A mechanism for ATP release was eluded to using Pannexin 1 knockout mice. Previous publications in epithelial cells have indicated that activation of TRPV4 can lead to ATP release via signalling mechanisms which lead to the opening of the Pannexin 1 pore (Seminario-Vidal *et al.*, 2011; Baxter *et al.*, 2014). Using *Px1^{-/-}* mice, it was demonstrated that GSK101 induced depolarisation was inhibited in tissue from these mice, whereas responses to capsaicin and acrolein remained unchanged.

In vivo, AF-353 (10mg/kg) inhibited both GSK101 (100ng/ml) and $\alpha\beta$ Me-ATP (300 μ M) induced firing of single airway terminating A δ -fibres in the guinea pig. However, use of the TRPV4 antagonist GSK2193874 in the same preparation inhibited GSK101 but not $\alpha\beta$ Me-ATP induced firing indicating that activation of P2X3 by ATP is downstream of activation of TRPV4. Furthermore, despite inhibiting firing induced by TRPV4, AF-353 had no effect on GSK101 induced bronchoconstriction, which was blocked by GSK2193874, indicating that GSK101 induced bronchoconstriction is independent of P2X3. This is to be expected as P2X3 is predominantly expressed on sensory neurons and very little is expressed on airway smooth muscle (Chen *et al.*, 1995; Burnstock, 2008). However, as the vagal nerves were cut, this does not rule out a role for P2X3 in reflex induced bronchoconstriction induced by TRPV4. Finally, AF-353 was also shown to inhibit GSK101 induced cough in the conscious guinea pig. These findings tie in with previous work that showed that $\alpha\beta$ Me-ATP causes firing of nodose, but not jugular derived C fibres (Undem 2004). This was attributed to the fact that jugular neurons predominantly expressed P2X3 but nodose fibres expressed more of the P2X2/3 heterodimers (Kwong *et al.*, 2008).

To conclude, the data within this thesis has outlined a novel role for a more mechanosensitive ion channel in the afferent arm of airway reflexes. Activation of TRPV4 by synthetic ligands and by hypoosmolarity was shown to cause activation of airway specific sensory nerves both *in vivo* and *in vitro* through activating nodose derived A δ airway fibres, a different mechanism to the well-known pro-tussive ion channels TRPV1 and TRPA1. This activation is proposed to be through release of ATP and subsequent activation of P2X3 present on the same subset of sensory neurons. In the lung, TRPV4 is likely to be acting as a cell sensor, where activators such as cell stretch, a hypotonic environment, or 5,6-EET, an arachidonic acid derivative, which are all indicative of disease conditions in the lung, lead to activation of TRPV4, and ATP

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is released as part of a cell signalling mechanism (**Figure 6.1**). This is important in terms of airway diseases such as asthma and COPD, where patients have been shown to have elevated levels of ATP in the airways (Mohsenin and Blackburn, 2006; Polosa and Holgate, 2006; Idzko *et al.*, 2007; Lommatzsch *et al.*, 2010) and also sensory nerve activity is enhanced.

Therapeutically, targeting the P2X3 arm of this pathway has already shown some beneficial effects in the clinic, where a P2X3 antagonist AF-219, which is structurally similar to the antagonist AF-353 used in this thesis, significantly inhibited daytime objective cough frequency in patients with idiopathic chronic cough (Abdulqawi *et al.*, 2014). However, the dose of the P2X3 antagonist AF-219 used (600mg twice a day) was associated with a loss of taste, which led to several participants leaving and also the unmasking of the study. The authors suggest that perhaps a lower concentration of the antagonist would bypass this side effect as 600mg of AF-219 shows occupancy at both homomeric P2X3 and the heterotrimeric P2X2/3 receptor, and it is the P2X2/3 heterodimer which has been implicated in perception of taste in rats and mice (Bo *et al.*, 1999; Finger *et al.*, 2005). However, if a TRPV4 antagonist was used instead, this would bypass any taste disturbance but still would potentially inhibit cough through blocking the release of ATP. Further it has been shown that AF-353 had no effect on TRPV4 mediated bronchoconstriction in the absence of a reflex arc. As TRPV4 activation has been shown to have profound effects on both bronchoconstriction and fibre firing in the lung it would perhaps be advantageous to also target TRPV4 therapeutically for treatment in whole lung disease. However further investigation into the mechanisms of bronchoconstriction are required.

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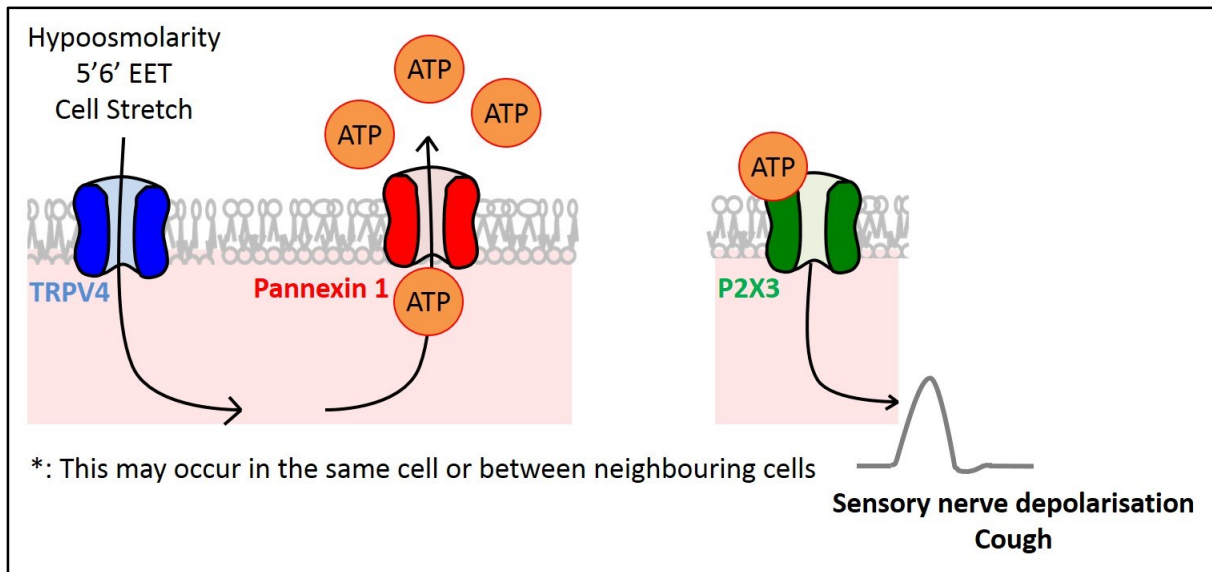


Figure 6.1: Proposed hypothesis for TRPV4 mediated ATP release and subsequent autocrine or paracrine activation of P2X3

The TRPV4 ion channel is activated by a number of ligands found in the diseases airway, including a reduced osmolarity and arachidonic acid derivatives. This causes a change in $[Ca^{2+}]_i$ signal which subsequently leads to ATP release, perhaps through activation of RhoA, which opens the Pannexin 1 ion channel. ATP may be acting in either an autocrine or paracrine fashion. This then causes activation of P2X3 which leads to sensory nerve depolarisation and airway reflexes such as cough.

6.2: Limitations of thesis

There are a number of limitations to the techniques used within this thesis, some of which have been outlined previously. The isolated vagus technique, although high throughput and readily amenable to pharmacology, is not a true measure of how events occur *in vivo*. Receptors and ion channels present on the nerve trunk may not be present in the same conformation as found at the nerve terminals, and the receptors and signalling proteins present on guinea pig and murine nerves may differ to those found in human tissue. Finally the vagus nerve innervates a number of midline organs and tissues alongside the lungs, therefore it cannot be confirmed that recordings taken are from airway specific fibres.

These particular issues were circumvented, where possible, by repeating experiments in donor human vagus to ensure that any results seen are likely to be translatable to the clinic. Furthermore responses were repeated in airway terminating single fibres in guinea pigs and also in an *in vivo* cough model, where responses involve interactions at the nerve terminals and are therefore more indicative of the reflex itself. Findings were also mirrored in guinea pig airway specific ganglia stained with the retrograde tracer dye Dil, and also in the guinea pig *in vivo* airway terminating single fibre technique, to ensure that responses seen were airway

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specific. However despite circumventing some issues found with the isolated vagus, these techniques also have mitigating issues of their own. The single fibre technique is both labour intensive and costly as only one fibre can be used at one time. In addition, there is currently no human model.

For TRPV4 and P2X3 expression, we have used whole vagal ganglia from the guinea pig. The whole ganglia, along with containing neurons also contain a dense vasculature, macrophages, cells of dendritic phenotype, and glial cells, therefore expression levels seen may not be fully representative of what is seen in the neurons. This could be circumvented by utilising single cell PCR. This involves picking single airway labelled jugular and nodose derived neurons and using a specialised protocol, measuring the RNA levels of targets within these cells. This technique has become fairly well established (Nassenstein *et al.*, 2010) and would therefore provide a more accurate picture of TRPV4 and P2X3 expression in neurons. In addition, similar to the single fibre experiments, there is currently no human model of functional imaging of vagal ganglia neurons as they are not easily available. This is likely to be due to their location (behind the ear in the skull). Although human dorsal root ganglia (DRGs), which convey somatic sensation at the spinal level to the CNS, are more readily available, these are mainly neural crest derived (Eng *et al.*, 2007). Although this is the same embryological origin as the jugular ganglia, the nodose ganglia are epibranchial placode derived (Nasra and Belvisi, 2009) and therefore the DRGs may not house a fully representative subset of nerve fibres. The *in vivo* cough study is also costly both in terms of compound and number of animals required.

6.3: Future work

6.3.1: Investigate the effects of TRPV4 and P2X3 antagonists on hypoosmolar induced responses *in vivo*

As shown in **Chapter 4**, hypoosmolar solutions caused firing of airway terminating A δ -fibres, however due to time constraints the responses were not tested against specific antagonists. Alongside testing the selective TRPV4 antagonist GSK2193874, it would also be interesting to investigate the role of the P2X3 antagonist AF-353 as hypoosmotic responses were inhibited by AF-353 *in vitro* in the isolated vagal nerve. A submaximal concentration of hypoosmotic solution (-60mOsm) would be utilised and two reproducible responses carried out. The animal would then be treated with AF-353 (10mg/kg), GSK2193874 (300mg/kg) or appropriate vehicle for the antagonist used and responses to -60mOsm repeated and responses monitored.

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Alongside investigating the effects of hypoosmolar solution in airway terminating single fibres, it would also be interesting to investigate the effect of hypoosmolar solutions on the cough response in guinea pigs. Aerosolised distilled water, a hypoosmotic solution, has been shown to induce cough in provocation studies in humans (Eschenbacher *et al.*, 1984; Fuller and Collier, 1984). A concentration response to hypoosmotic solution would be carried out in guinea pigs and a submaximal concentration used against TRPV4 or P2X3 antagonists or appropriate vehicle to determine if the same mechanism as for TRPV4 induced responses is also applicable for hypoosmolar induced responses *in vivo*.

6.3.2: Investigate the effects of TRPV4 and P2X3 in disease models

All experiments within this thesis were carried out in naïve animals as it is necessary to understand the role of TRPV4 in the acute cough response. However, to further add to understanding of the role of TRPV4 in the lung it would also be important to investigate whether TRPV4 and P2X3 play a role in the modification of the cough response in disease states using *in vivo* model systems. Endogenous ligands of the TRPV4 ion channel are including arachidonic acid derivatives and osmotic stimuli (Watanabe *et al.*, 2003; Jia *et al.*, 2004; Vriens *et al.*, 2005) have been shown to be released or altered in the diseased and inflamed airway (Joris *et al.*, 1993, Rolin *et al.*, 2005, Naruyima *et al.*, 1999). Furthermore, as mentioned previously, increased ATP levels have been found in the airways of patients with asthma and COPD (Mohsenin and Blackburn, 2006; Polosa and Holgate, 2006; Idzko *et al.*, 2007; Lommatzsch *et al.*, 2010; Mortaz *et al.*, 2010), and AF-219 was shown to inhibit chronic cough suggesting a role for ATP in patients with chronic cough (Abdulqawi *et al.*, 2014). Taken together this would suggest that TRPV4 could play a role in the diseased airway.

Previous work has shown that vagal nerve responses and cough responses to TRPV1 have been shown to be enhanced (Wortley *et al.*, 2014) in a guinea pig cigarette smoke model which mimics the effects of COPD. In order to determine if TRPV4 and P2X3 responses are also enhanced, an *in vivo* model of cigarette smoke exposed guinea pigs would be used. Guinea pigs would be exposed to cigarette smoke for one hour twice a day for 8 days to mimic the inflammatory response seen in the airways in COPD. Vagal nerve tissue can then be taken from these animals and responses to both TRPV4 and P2X3 ligands compared to an air exposed control group, or alternatively a cough provocation study could be carried out comparing responses to cigarette smoke exposed and air exposed animals. This would allow the investigation of whether or not TRPV4 and P2X3 responses are enhanced under disease conditions caused by exposure to cigarette smoke.

Further, as asthmatics have been shown to cough to ATP (Basoglu *et al.*, 2005), and in guinea pigs, following ATP administration the number of coughs to citric acid was significantly

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enhanced (Kamei *et al.*, 2005), the role of TRPV4 could also be investigated in asthma-driven responses. A model of OVA sensitised guinea pigs could be utilised (Delescluse *et al.*, 2012), where guinea pigs are sensitised with OVA or vehicle on days 0 and 7 and challenged on day 28. Tissues and responses would be taken and monitored 10 days after challenge. Similarly to above, vagal nerve tissue could be taken from OVA challenged animals and responses to TRPV4 and P2X3 agonist compared to those of saline treated animals. In addition, a cough provocation study could be carried out investigating whether or not TRPV4 mediated cough responses were enhanced.

6.3.2: Investigate a Source of ATP

In order to fully confirm that TRPV4 is causing ATP release, and to confirm the cell type responsible, an ATP release assay could be carried out. Neurones from the nodose ganglia, which are known to be activated by the TRPV4 agonist GSK101, could be isolated from all other cell types, including satellite cells and glial cells, and stimulated with GSK101. Following a set time period, the cell supernatant would be removed and ATP levels measured using an ATPlite luminescence detection system (Perkin Elmer, Cambridge, UK) as described in (Baxter *et al.*, 2014). This assay utilises luciferase which catalyses the reaction of D-luciferin with the ATP present to produce oxyluciferin and light, where the amount of light is proportional to the amount of ATP present. ATP release could then be determined to occur from the ganglia itself, or if not, from surrounding cells and tissues when activated by the TRPV4 agonist.

6.3.3: Investigate the effects of TRPV4 ligands on bronchoconstriction

In [Chapter 3](#) it was shown that firing induced by GSK101 was accompanied by a significant and sustained bronchoconstriction in all animals investigated which, similarly to the firing seen, was delayed in onset, but lasted for over 30 minutes. It has previously been shown that 4αPDD and hypoosmotic solutions cause bronchoconstriction in isolated human airway smooth muscle (Jia *et al.*, 2004). Preliminary data has shown that this bronchoconstriction was mimicked in both guinea pig and human tissue, which was blocked in a concentration dependent manner by the TRPV4 antagonist GSK2193874 (**Figure 6.2**). Publications have attributed the contraction seen to the release of cysteinyl leukotrienes (McAlexander *et al.*, 2014).

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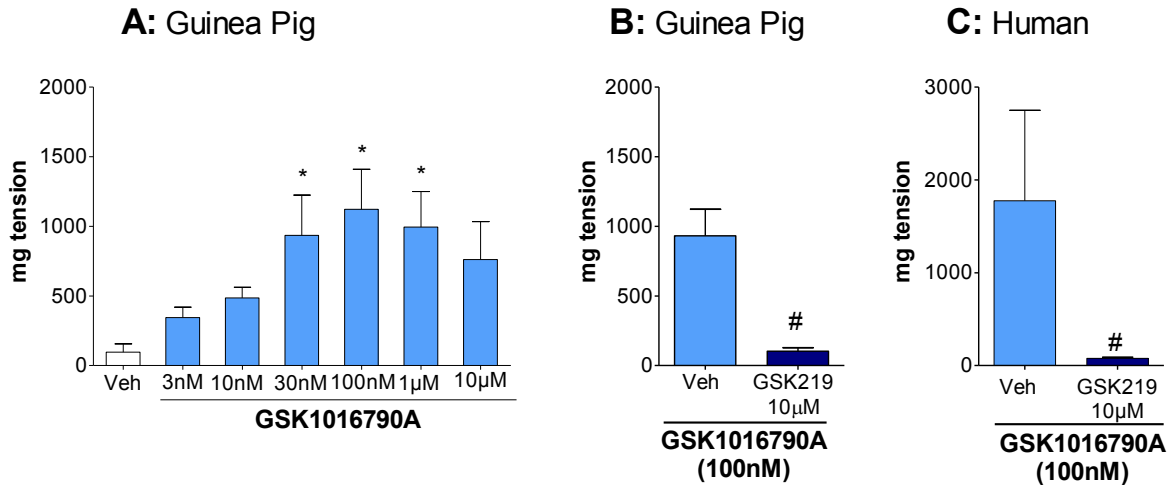


Figure 6.2: The role of TRPV4 in bronchoconstriction

A: The TRPV4 agonist GSK101 caused a concentration dependent increase in contraction in the guinea pig trachea. This was significantly inhibited by the TRPV4 antagonist GSK219 (10µM) in both guinea pig (B) and human (C) tissue. Data shown as mean±SEM of n=4-6 (guinea pig) and n=3 (human). * indicates significance at $p > 0.05$, one way ANOVA with Dunns post-test comparing responses to vehicle, # indicates statistical significance at $p > 0.05$ paired t-test. Human donor tissue was obtained from 2 male and 1 female donors aged 45-70 with no known respiratory disease.

P2X3 is expressed predominantly on sensory nerves, and there is no reported expression in airway smooth muscle (Burnstock, 2008). Furthermore, as shown in **Chapter 5**, GSK101 induced contraction was not inhibited by the P2X3 antagonist AF-353 *in vivo*, which is expected as the vagus nerve was cut. However it does not rule out the role of P2X3 in reflex bronchoconstriction. McAlexander *et al* have eluded to a possible mechanism involved in contraction, suggesting the involvement of mast cells and subsequent release of cysteinyl leukotrienes which causes contraction. This was shown as GSK101 induced contraction was blocked by the CystLt receptor antagonist montelukast (McAlexander *et al.*, 2014).

Preliminary data has shown that TRPV4 does not seem to be expressed on human lung mast cells (**Figure 6.3**), indicating that the release of cysteinyl leukotrienes is not likely to be a direct effect of TRPV4 agonists acting on mast cells. Instead, as suggested by the slow onset of action, it could occur through the release of a secondary mediator. As it has been shown in this thesis, TRPV4 activation of sensory nerves causes ATP release, as does activation of epithelial cells (Baxter *et al.*, 2014). Therefore it could be assumed that the mechanisms involved in bronchoconstriction could also involve the release of ATP. In order to investigate this further, initially ATP release could be measured from isolated human airway smooth muscle cells following administration of a TRPV4 agonist. In addition, expression of P2X1, P2X4 and P2X7 receptors have been shown on human lung mast cells (Wareham *et al.*, 2009)

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and their role in GSK101 induced contraction could be investigated. This will help to uncover the mechanism of action behind the sustained bronchoconstriction seen both *in vitro* and *in vivo*.

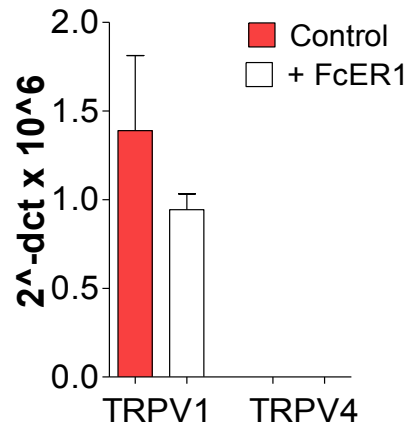


Figure 6.3: TRPV4 expression in Human Lung Mast Cells

Relative expression of TRPV1 (control) and TRPV4 mRNA in human lung mast cells (HLMCs). N=3 for control and n=3 for FcER1.

6.3.4: Clinical Studies

The ultimate aim of this work would be to take it forward into a small proof of concept study in patients with chronic cough. The P2X3 antagonist AF-219 has already shown efficacy in patients with chronic cough, but activation of P2X3 has shown no effect on bronchospasm in the guinea pig. As activation of TRPV4 causes both nerve firing and bronchoconstriction, targeting TRPV4 could be a more global target for the treatment of lung disease. An oral TRPV4 antagonist GSK2798745 is currently being used in a placebo controlled Phase 1 clinical trial to investigate the safety, tolerability, pharmacokinetics, and pharmacodynamics of repeat doses of the drug in healthy controls and patients with stable heart failure (Clinical trial identifier: NCT02119260). If shown to be safe and well tolerated, this compound could also be taken forward into patients with airway disease and associated chronic cough.

6.4: Concluding Remarks

The work carried out for this thesis has shown that TRPV4 causes activation of sensory nerves by activating nodose derived A δ -fibres, a different mechanism to that seen with activation of the well-known TRP channels TRPV1 and TRPA1. This activation has been shown to be through release of ATP and subsequent activation of P2X3 receptors on potentially the same subset of nerve fibres. A role for TRPV4 in the activation of airway sensory nerves and cough has therefore been determined, and a possible mechanism of action has also been outlined, and therefore the original thesis hypothesis can be accepted. The future work that has been outlined above should help to further this knowledge and also uncover a potential role of TRPV4 in disease.

The aim of this research was to understand the role of TRPV4 in the cough reflex and potentially uncover a novel therapeutic target for the treatment of cough, which is currently an urgent unmet medical need. Chronic cough is associated with the inflammatory diseases asthma and COPD, where levels of ATP are known to be elevated (Mohsenin and Blackburn, 2006; Polosa and Holgate, 2006; Idzko *et al.*, 2007; Lommatzsch *et al.*, 2010; Mortaz *et al.*, 2010) and patients have shown increased cough, dyspnea and chest tightness in response to exogenous ATP (Basoglu *et al.*, 2005, Basoglu *et al.*, 2015). Furthermore, there are polymorphisms in the TRPV4 gene which are associated with COPD (Zhu *et al.*, 2009). Taken together, this indicates that elevated ATP could be a key driver in the pathogenesis of cough in asthma and COPD, and targeting the TRPV4 ion channel could be an attractive target for treatment.

This is the first primarily mechanosensitive ion channel that has been implicated in afferent arm of the reflex and the cough response, and therefore antagonists may aid to treat patients where TRPA1 and TRPV1 antagonists are less effective. Furthermore, through investigation of the mechanism of TRPV4 induced bronchoconstriction, this may uncover a novel mechanism of action which can be targeted in disease. Together with the implicated role of TRPV4 in inflammation (Baxter *et al.*, 2014), and a recently discovered role in fibrosis (Rahaman *et al.*, 2014), this would suggest a more global role for TRPV4 in the airways, and makes the ion channel an attractive new global therapeutic target for airway disease.

Chapter 7:

Bibliography

- Abdulqawi R, Dockry R, Holt K, Layton G, McCarthy BG, Ford AP, and Smith JA (2014) P2X3 receptor antagonist (AF-219) in refractory chronic cough: a randomised, double-blind, placebo-controlled phase 2 study. *Lancet* **14**:61255-1.
- Abe K, Watanabe N, Kumagai N, Mouri T, Seki T, and Yoshinaga K (1967) Circulating kinin in patients with bronchial asthma. *Experientia* **23**:626–627.
- Adcock JJ (2009) TRPV1 receptors in sensitisation of cough and pain reflexes. *Pulm Pharmacol Ther* **22**:65–70
- Adcock JJ, Birrell MA, Maher SA, Bonvini SJ, Dubuis ED, Wortley MA, and Baker KE (2014) Making Sense Of Sensory Nerves: An In Vivo Characterisation Of Aδ- And C-Fibres Innervating Guinea-Pig Airways. *AJRCCM* A3969.
- Adcock JJ, Douglas GJ, Garabette M, Gascoigne M, Beatch G, Walker M, and Page CP (2003) RSD931, a novel anti-tussive agent acting on airway sensory nerves. *Br J Pharmacol* **138**:407–416.
- Adriaensen D, and Timmermans JP (2004) Purinergic signalling in the lung: important in asthma and COPD? *Current Opinion Pharmacology* **4**:207-14
- Aizawa N, Wyndaele J, Homma Y, and Igawa Y (2012) Effects of TRPV4 Cation Channel Activation on the Primary Bladder Afferent Activities of the Rat. *Neurology and Urodynamics* **155**:148–155.
- Alessandri-Haber N, Dina OA, Joseph EK, Reichling D, and Levine JD (2006) A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J Neurosci* **26**:3864–74.
- Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, and Levine JD (2005) TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. *Pain* **118**:70–9.
- Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, and Levine JD (2003) Hypotonicity induces TRPV4-mediated nociception in rat. *Neuron* **39**:497–511.
- Alvarez DF, King JA, Weber D, Addison E, Liedtke W, and Townsley MI (2006) Transient receptor potential vanilloid 4-mediated disruption of the alveolar septal barrier: a novel mechanism of acute lung injury. *Circ Res* **99**:988–995.
- Amadesi S, Nie J, Vergnolle N, Cottrell GS, Grady EF, Trevisani M, Manni C, Geppetti P, McRoberts JA, Ennes H, Davis JB, Mayer EA, and Bunnett NW (2004) Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *J Neurosci* **24**:4300–12.
- Anderson SD, and Kippelen P (2012) Assessment and prevention of exercise-induced bronchoconstriction. *Br J Sports Med* **46**: 391-396
- Anderson SD, Pearlman DS, Rundell KW, Perry CP, Boushey H, Sorkness CA, Nichols S, and Weiler JM (2010) Reproducibility of the airway response to an exercise protocol standardized for intensity, duration, and inspired air conditions, in subjects with symptoms suggestive of asthma. *Respir Res* **11**:120.

- Andersson DA, Gentry C, Moss S, and Bevan S (2008) Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci* **28**:2485–94.
- Andr  E, Campi B, Materazzi S, Trevisani M, Amadesi S, Massi D, Creminon C, Vaksman N, Nassini R, Civelli M, Baraldi PG, Poole DP, Bunnett NW, Geppetti P, and Patacchini R (2008) Cigarette smoke–induced neurogenic inflammation is mediated by α , β -unsaturated aldehydes and the TRPA1 receptor in rodents. *J. Clin. Invest.* **118**: 2574–82.
- Andr  E, Gatti R, Trevisani M, Preti D, Baraldi PG, Patacchini R, Geppetti P, and Andre E (2009) Transient receptor potential ankyrin receptor 1 is a novel target for pro-tussive agents. *Br J Pharmacol* **158**:1621–1628.
- Baiardini I, Braido F, Fassio O, Tarantini F, Pasquali M, Tarchino F, Berlendis A, and Canonica GW (2005) A new tool to assess and monitor the burden of chronic cough on quality of life: Chronic Cough Impact Questionnaire. *Allergy* **60**:482–488
- Baluk P, Nadel JA, and McDonald DM (1992) Substance P-immunoreactive sensory axons in the rat respiratory tract: a quantitative study of their distribution and role in neurogenic inflammation. *J Comp Neurol* **319**:586–98.
- Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, and Patapoutian A (2004) Noxious Cold Ion Channel TRPA1 Is Activated by Pungent Compounds and Bradykinin. *Neuron* **41**:849–857.
- Banner KH, Igney F, and Poll C (2011) TRP channels: emerging targets for respiratory disease. *Pharmacol Ther* **130**:371–84
- Bao L, Locovei S, and Dahl G (2004) Pannexin membrane channels are mechanosensitive conduits for ATP. *FEBS Lett* **572**:65–68.
- Bargiotas P, Krenz A, Hormuzdi SG, Ridder DA, Herb A, and Barakat W (2011) Pannexins in ischemia-induced neurodegeneration. *Proc Natl Acad Sci* **108**: 20772–20777
- Barnes PJ (2013) Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* **131**:636–645
- Barnes PJ (2008) Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* **8**:183–192
- Barry SJ, Dane AD, Morice AH, and Walmsley AD (2006) The automatic recognition and counting of cough. *Cough* **2**:8.
- Bartlett D, Jeffery P, Sant’Ambrogio G, and Wise JC (1976) Location of stretch receptors in the trachea and bronchi of the dog. *J Physiol* **258**:409–420.
- Basoglu OK, Pelleg A, Essilfie-Quaye S, Brindicci C, Barnes PJ, and Kharitonov SA (2005) Effects of aerosolized adenosine 5-triphosphate vs adenosine 5-monophosphate on dyspnea and airway caliber in healthy nonsmokers and patients with asthma. *Chest* **128**:1905–1909.
- Basoglu OK, Barnes PJ, Kharitonov SA, and Pelleg A (2015) Effects of aerosolized adenosine 5-triphosphate in smokers and patients with chronic obstructive pulmonary disease. *Chest*. **14**:2285

- Bateman ED, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE, and Zar HJ (2008) Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J Off J Eur Soc Clin Respir Physiol* **31**:143–178.
- Bautista DM (2005) Pungent products from garlic activate the sensory ion channel TRPA1. *Proc Natl Acad Sci* **102**:12248–12252
- Bautista DM, Jordt S-E, Nikai T, Tsuruda PR, Read AJ, Poblete J, Yamoah EN, Basbaum AI, and Julius D (2006) TRPA1 Mediates the Inflammatory Actions of Environmental Irritants and Proalgesic Agents. *Cell* **124**:1269–1282.
- Bautista DM, Pellegrino M, and Tsunozaki M (2013) TRPA1: A gatekeeper for inflammation. *Annu Rev Physiol* **75**:181–200.
- Baxter M, Eltom S, Dekkak B, Yew-Booth L, Dubuis E, Maher SA, Belvisi MG, and Birrell MA (2014) Role of transient receptor potential and pannexin channels in cigarette smoke-triggered ATP release in the lung. *Thorax* **60**. doi:10.1136/thoraxjnl-2014-205467
- Belvisi MG (2002) Overview of the innervation of the lung. *Curr Opin Pharmacol* **2**:211–215.
- Belvisi MG (2003) Sensory nerves and airway inflammation: role of A δ and C-fibres. *Pulm Pharmacol Ther* **16**:1–7.
- Belvisi MG (2014) Therapeutic advances for treatment resistant cough. *Lancet* **385**: 1160-2
- Belvisi MG, Dubuis E, and Birrell MA (2011) Transient Receptor Potential A1 Channels: Insights Into Cough and Airway Inflammatory Disease. *Chest* **140**:1040–1047,
- Belvisi MG, and Geppetti P (2004) Cough. 7: Current and future drugs for the treatment of chronic cough. *Thorax* **59**:438–40.
- Belvisi MG, and Hele DJ (2003) Soft steroids: a new approach to the treatment of inflammatory airways diseases. *Pulm Pharmacol Ther* **16**:321–5.
- Bessac BF, and Jordt S-E (2008) Breathtaking TRP Channels: TRPA1 and TRPV1 in Airway Chemosensation and Reflex Control. *Physiology* **23**:360–370.
- Bhowmik A, Seemungal TA, Sapsford RJ, and Wedzicha JA (2000) Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* **55**:114–120
- Bianco S, Vaghi A, Robuschi M, and Pasargiklian M (1988) Prevention of exercise-induced bronchoconstriction by inhaled frusemide. *Lancet* **2**:252–255.
- Biernacki WA, Kharitonov SA, and Barnes PJ (2003) Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax* **58**:294–298
- Birder L, Kullmann FA, Lee H, Barrick S, de Groat W, Kanai A, and Caterina M (2007) Activation of urothelial transient receptor potential vanilloid 4 by 4 α -phorbol 12,13-didecanoate contributes to altered bladder reflexes in the rat. *J Pharmacol Exp Ther* **323**:227–235.

- Birrell MA, Belvisi MG, Grace M, Sadofsky L, Faruqi S, Hele DJ, Maher SA, Freund-Michel V, and Morice AH (2009) TRPA1 Agonists Evoke Coughing in Guinea Pig and Human Volunteers. *Am J Respir Crit Care Med* **180**:1042–1047.
- Birrell MA, Bonvini SJ, Dubuis E, Maher SA, Wortley MA, Grace MS, Raemdonck K, Adcock JJ, and Belvisi MG (2014) Tiotropium modulates transient receptor potential V1 (TRPV1) in airway sensory nerves: A beneficial off-target effect?*. *J Allergy Clin Immunol* **3**:679–687
- Birrell MA, Crispino N, Hele DJ, Patel HJ, Yacoub MH, Barnes PJ, and Belvisi MG (2002) Effect of dopamine receptor agonists on sensory nerve activity: possible therapeutic targets for the treatment of asthma and COPD. *Br J Pharmacol* **136**:620–628.
- Birring SS (2011) Controversies in the evaluation and management of chronic cough. *AJRCCM* **183**:708–715
- Birring SS, Brightling CE, Symon FA, Barlow SG, Wardlaw AJ, and Pavord ID (2003) Idiopathic chronic cough: association with organ specific autoimmune disease and bronchoalveolar lymphocytosis. *Thorax* **58**:1066–1070.
- Birring SS, Fleming T, Matos S, Raj AA, Evans DH, and Pavord ID (2008) The Leicester Cough Monitor: preliminary validation of an automated cough detection system in chronic cough. *Eur Respir J* **31**:1013–1018
- Birring SS, Matos S, Patel RB, Prudon B, Evans DH, and Pavord ID (2006) Cough frequency, cough sensitivity and health status in patients with chronic cough. *Respir Med* **100**:1105–1109
- Birring SS, Parker D, Brightling CE, Bradding P, Wardlaw AJ, and Pavord ID (2004) Induced sputum inflammatory mediator concentrations in chronic cough. *Am J Respir Crit Care Med* **169**:15–19.
- Birring SS, Prudon B, Carr AJ, Singh SJ, Morgan MDL, and Pavord ID (2003) Development of a symptom specific health status measure for patients with chronic cough: Leicester Cough Questionnaire (LCQ). *Thorax* **58**:339–343
- Bo X, Alavi A, Xiang Z, Oglesby I, Ford A and Burnstock G (1999) Localization of ATP-gated P2X2 and P2X3 receptor immunoreactive nerves in rat taste buds *Neuroreport* **10**: 1107–11
- Bolser DC (2006) Cough suppressant and pharmacologic protussive therapy: ACCP evidence-based clinical practice guidelines. *Chest* **129**:238S–249S
- Bongianni F, Mutolo D, Fontana GA, and Pantaleo T (1998) Discharge patterns of Bötzing complex neurons during cough in the cat. *Am J Physiol* **274**:R1015–24
- Bonini S, Lambiase A, Bonini S, Angelucci F, Magrini L, Manni L, and Aloe L (1996) Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci U S A* **93**:10955–10960.
- Bonvini SJ, Birrell MA, Smith JA, and Belvisi MG (2015) Targeting TRP channels for chronic cough: from bench to bedside. *Naunyn Schmiedebergs Arch Pharmacol* **388**: 401–20

- Bourque CW, and Oliet SH (1997) Osmoreceptors in the central nervous system. *Annu Rev Physiol* **59**:601–19.
- Brignall K, Jayaraman B, and Birring SS (2008) Quality of life and psychosocial aspects of cough. *Lung* **186** Suppl:S55–8.
- Brouns I, Adriaensen D, Burnstock G, and Timmermans JP (2000) Intraepithelial vagal sensory nerve terminals in rat pulmonary neuroepithelial bodies express P2X3 receptors. *Am J Respir Cell Mol Biol* **23**:52–61.
- Brouns I, Pintelon I, Timmermans JP, and Adriaensen D (2012) Novel insights in the neurochemistry and function of pulmonary sensory receptors. *Adv Anat Embryol Cell Biol* **211**:1–115, vii.
- Brozmanova M, Mazurova L, Ru F, Tatar M, and Kollarik M (2012) Comparison of TRPA1-versus TRPV1-mediated cough in guinea pigs. *Eur J Pharmacol* **689**:211–8.
- Burnstock G (2001) Purine-mediated signalling in pain and visceral perception. *Trends Pharmacol Sci* **22**:182–188.
- Burnstock G (2008) Purinergic signalling and disorders of the central nervous system. *Nat Rev Drug Discov* **7**:575–590.
- Burnstock G, and Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoreceptor? *Gen Pharmacol* **16**:433–440.
- Burnstock G, and Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *International Review of Cytology* **240**: 31-304
- Burnstock G, and Verkhatsky A (2010) Long-term (trophic) purinergic signalling: purinoreceptors control cell proliferation, differentiation and death. *Cell Death Dis* **1**:e9.
- Canning BJ, Mori N, and Mazzone SB (2006a) Vagal afferent nerves regulating the cough reflex. *Respir Physiol Neurobiol* **152**:223–242
- Canning BJ (2006b) Anatomy and neurophysiology of the cough reflex: ACCP evidence-based clinical practice guidelines. *Chest* **129**:33S–47S.
- Canning BJ (2009) Central regulation of the cough reflex: therapeutic implications. *Pulm Pharmacol Ther* **22**:75–81.
- Canning BJ (2011) Functional implications of the multiple afferent pathways regulating cough. *Pulm Pharmacol Ther* **24**:295–9.
- Canning BJ (2004) Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs. *J Physiol* **557**:543–558
- Canning BJ, and Mori N (2011) Encoding of the cough reflex in anesthetized guinea pigs. *Am J Physiol - Regul Integr Comp Physiol* **300**:R369–R377
- Canning BJ, and Mori N (2011) Encoding of the cough reflex in anesthetized guinea pigs. *Am J Physiol Regul Integr Comp Physiol* **300**:R369–77.

- Capasso R, Aviello G, Romano B, Borrelli F, De Petrocellis L, Di Marzo V, and Izzo AA (2012) Modulation of mouse gastrointestinal motility by allyl isothiocyanate, a constituent of cruciferous vegetables (Brassicaceae): evidence for TRPA1-independent effects. *Br J Pharmacol* **165**:1966–77.
- Carr MJ, Hunter DD, Jacoby DB, and Undem BJ (2002) Expression of Tachykinins in Nonnociceptive Vagal Afferent Neurons during Respiratory Viral Infection in Guinea Pigs. *AJRCCM* **8**:1071-1075.
- Carr MJ, and Undem BJ (2003) Pharmacology of vagal afferent nerve activity in guinea pig airways. *Pulm Pharmacol Ther* **16**:45–52.
- Caterina MJ (2000) Impaired Nociception and Pain Sensation in Mice Lacking the Capsaicin Receptor. *Science* **288**:306–313.
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**:816–824
- Centers for Disease Control and Prevention (2007) Infant deaths associated with cough and cold medications--two states, 2005. *MMWR Morb Mortal Wkly Rep* **56**:1–4.
- Chaudhuri R, McMahon AD, Thomson LJ, MacLeod KJ, McSharry CP, Livingston E, McKay A, and Thomson NC (2004) Effect of inhaled corticosteroids on symptom severity and sputum mediator levels in chronic persistent cough. *J Allergy Clin Immunol* **113**:1063–1070.
- Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, and Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. *Nature* **377**:428–431.
- Cheney FW, and Butler J (1968) The effects of ultrasonically-produced aerosols on airway resistance in man. *Anesthesiology* **29**:1099–1106.
- Chizh BA, and Sang CN (2009) Use of sensory methods for detecting target engagement in clinical trials of new analgesics. *Neurotherapeutics* **6**:749–54.
- Chou Y-L, Scarupa MD, Mori N, and Canning BJ (2008) Differential effects of airway afferent nerve subtypes on cough and respiration in anesthetized guinea pigs. *Am J Physiol Regul Integr Comp Physiol* **295**:R1572–84.
- Choudry NB, and Fuller RW (1992) Sensitivity of the cough reflex in patients with chronic cough. *Eur Respir J* **5**:296–300
- Choudry NB, Fuller RW, and Pride NB (1989) Sensitivity of the human cough reflex: effect of inflammatory mediators prostaglandin E2, bradykinin, and histamine. *Am Rev Respir Dis* **140**:137–141.
- Chuaychoo B, Hunter DD, Myers AC, Kollarik M, and Undem BJ (2005) Allergen-induced substance P synthesis in large-diameter sensory neurons innervating the lungs. *J Allergy Clin Immunol* **116**:325–331
- Chung KF (2011) Chronic “cough hypersensitivity syndrome”: A more precise label for chronic cough. *Pulm Pharmacol Ther* **24**:267–271

- Chung KF, and Pavord ID (2008) Chronic Cough 1 Prevalence , pathogenesis , and causes of chronic cough. *The Lancet*: **371**: 1364-1374
- Ciura S, and Bourque CW (2006) Transient receptor potential vanilloid 1 is required for intrinsic osmoreception in organum vasculosum lamina terminalis neurons and for normal thirst responses to systemic hyperosmolality. *J Neurosci* **26**:9069–75.
- Ciura S, Liedtke W, and Bourque CW (2011) Hypertonicity sensing in organum vasculosum lamina terminalis neurons: a mechanical process involving TRPV1 but not TRPV4. *J Neurosci* **31**:14669–76.
- Clapham DE (2003) TRP channels as cellular sensors. *Nature* **426**:517–524.
- Coleridge JC, and Coleridge HM (1984) Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Rev Physiol Biochem Pharmacol* **99**:1–110.
- Corrao WM, Braman SS, and Irwin RS (1979) Chronic cough as the sole presenting manifestation of bronchial asthma. *N Engl J Med* **300**:633–637.
- Cullinan P (1992) Persistent cough and sputum: prevalence and clinical characteristics in south east England. *Respir Med* **86**:143–149
- Curley FJ, Irwin RS, Pratter MR, Stivers DH, Doern G V, Vernaglia PA, Larkin AB, and Baker SP (1988) Cough and the common cold. *Am Rev Respir Dis* **138**:305–11.
- De Marco R, Marcon A, Jarvis D, Accordini S, Almar E, Bugiani M, Carolei A, Cazzoletti L, Corsico A, Gislason D, Gulsvik A, Jögi R, Marinoni A, Martínez-Moratalla J, Pin I, and Janson C (2006) Prognostic factors of asthma severity: A 9-year international prospective cohort study. *J Allergy Clin Immunol* **117**:1249–1256.
- Decalmer SC, Webster D, Kelsall AA, McGuinness K, Woodcock AA, and Smith JA (2007) Chronic cough: how do cough reflex sensitivity and subjective assessments correlate with objective cough counts during ambulatory monitoring? *Thorax* **62**:329–334
- Delescluse I, Mace H, and Adcock JJ (2012) Inhibition of airway hyper-responsiveness by TRPV1 antagonists (SB-705498 and PF-04065463) in the unanaesthetized, ovalbumin-sensitized guinea pig. *Br J Pharmacol* **166**:1822–32.
- Denton DA, McKinley MJ, and Weisinger RS (1996) Hypothalamic integration of body fluid regulation. *Proc Natl Acad Sci* **93**:7397–7404.
- Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, Ricciardolo FLM, Magno F, D'Anna SE, Zanini A, Carbone M, Sabatini F, Usai C, Brun P, Chung KF, Barnes PJ, Papi A, Adcock IM, and Balbi B (2009) Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax* **64**:968–975.
- Dicpinigaitis P V, and Alva R V (2005) Safety of capsaicin cough challenge testing. *Chest* **128**:196–202
- Dicpinigaitis P V (2007) Experimentally induced cough. *Pulm Pharmacol Ther* **20**:319–24.

- Dicpinigaitis PV (2006) Potential future therapies for the management of cough: ACCP evidence-based clinical practice guidelines. *Chest* **129**:169S–173S
- Dicpinigaitis PV (2011) Cough: an unmet clinical need. *Br J Pharmacol* **163**:116–24.
- Dicpinigaitis PV, Morice AH, Birring SS, Mcgarvey L, Smith JA, Canning BJ, and Page CP (2014) Antitussive Drugs — Past, Present, and Future. *Pharmacol Rev* **66**:468–512.
- Dietrich A, Chubakov V, Kalwa H, Rost BR, and Gudermann T (2006) Cation channels of the transient receptor potential superfamily: Their role in physiological and pathophysiological processes of smooth muscle cells. *Pharmacol Ther* **112**:744–760.
- Doherty MJ, Mister R, Pearson MG, and Calverley PM (2000a) Capsaicin responsiveness and cough in asthma and chronic obstructive pulmonary disease. *Thorax* **55**:643–649
- Doherty MJ, Mister R, Pearson MG, and Calverley PM (2000b) Capsaicin responsiveness and cough in asthma and chronic obstructive pulmonary disease. *Thorax* **55**:643–9.
- Dubuis E, Grace M, Wortley MA, Birrell MA, and Belvisi MG (2013) Harvesting, isolation, and functional assessment of primary vagal ganglia cells. *Curr Protoc Pharmacol* **62**:Unit 12.15.
- Eccles R (2006) Mechanisms of the placebo effect of sweet cough syrups. *Respir Physiol Neurobiol* **152**:340–8.
- Eid SR (2011) Therapeutic targeting of TRP channels--the TR(i)P to pain relief. *Curr Top Med Chem* **11**:2118–30.
- Elia C, Bucca C, Rolla G, Scappaticci E, and Cantino D (1988) A freeze-fracture study of human bronchial epithelium in normal, bronchitic and asthmatic subjects. *J Submicrosc Cytol Pathol* **20**:509–517.
- Eschenbacher WL, Boushey HA, and Sheppard D (1984) Alteration in osmolarity of inhaled aerosols cause bronchoconstriction and cough, but absence of a permeant anion causes cough alone. *Am Rev Respir Dis* **129**:211–215.
- Everaerts W, Nilius B, and Owsianik G (2010) The vanilloid transient receptor potential channel TRPV4: from structure to disease. *Prog Biophys Mol Biol* **103**:2–17
- Fahim A, Dettmar PW, Morice AH, and Hart SP (2011) Gastroesophageal reflux and idiopathic pulmonary fibrosis: a prospective study. *Medicina (Kaunas)* **47**:200–5.
- Faruqi S, Murdoch RD, Allum F, and Morice AH (2014) On the definition of chronic cough and current treatment pathways: an international qualitative study. *Cough* **10**:5.
- Fernández-Fernández JM, Andrade YN, Arniges M, Fernandes J, Plata C, Rubio-Moscardo F, Vázquez E, and Valverde MA (2008) Functional coupling of TRPV4 cationic channel and large conductance, calcium-dependent potassium channel in human bronchial epithelial cell lines. *Pflugers Arch* **457**:149–159.
- Finget TE, Danilova V, Barrows J, Bartell DL, Vigers AJ, Stone, L, Hellekant G and Kinnamon SC (2005) ATP signalling is crucial for communication from taste bud nerves *Science* **310**: 1495-1499

- Fong J, Sandhu G, Ellaway P, Davey N, Strutton P, Murphy K, and Guz A (2004) What do we know about how humans cough? *Pulm Pharmacol Ther* **17**:431–434
- Fontana GA, Pantaleo T, Lavorini F, Maluccio NM, Mutolo D, and Pistolesi M (1999) Repeatability of cough-related variables during fog challenges at threshold and suprathreshold stimulus intensity in humans. *Eur Respir J* **13**:1447–50.
- Fontana GA, Lavorini F, and Pistolesi M (2002) Water aerosols and cough. *Pulm Pharmacol Ther* **15**:205–211.
- Footitt J, and Johnston SL (2009) Cough and viruses in airways disease: Mechanisms. *Pulm Pharmacol Ther* **22**:108–113.
- Ford AP (2012) In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization. *Purinergic Signal* **8**:3–26.
- Ford AP, Gever JR, Nunn PA, Zhong Y, Cefalu JS, Dillon MP, and Cockayne DA (2006) Purinoceptors as therapeutic targets for lower urinary tract dysfunction. *Br J Pharmacol* **147**:S132–S143.
- Fox A, Barnes P, Urban L, and Dray A (1993) An in vitro study of the properties of single vagal afferents innervating guinea-pig airways. *J Physiol* **469**:21–35.
- Fox AJ, Barnes PJ, and Dray A (1995a) Stimulation of guinea-pig tracheal afferent fibres by non-isosmotic and low-chloride stimuli and the effect of frusemide. *J Physiol* **482** (Pt 1):179–87.
- Fox AJ, Belvisi MG, Fan K, Barnes PJ, Umesh G, Fox J, Belvisi G, and Chung F (1995b) Capsazepine inhibits cough induced and citric acid but not by hypertonic by capsaicin saline in guinea pigs. *Journal of Applied Physiology* **79**: 1082-1087
- Fox AJ, Urban L, Barnes PJ, and Dray A (1995c) Effects of capsazepine against capsaicin- and proton-evoked excitation of single airway C-fibres and vagus nerve from the guinea-pig. *Neuroscience* **67**:741–752.
- French CL (1998) Impact of Chronic Cough on Quality of Life. *Arch Intern Med* **158**:1657–1661.
- French CT, Irwin RS, Fletcher KE, and Adams TM (2002) Evaluation of a cough-specific quality-of-life questionnaire. *Chest* **121**:1123–1131.
- Fuller RW, and Choudry NB (1987) Increased cough reflex associated with angiotensin converting enzyme inhibitor cough. *Br Med J (Clin Res Ed)* **295**:1025–6.
- Fuller RW, and Collier JG (1984) Sodium cromoglycate and atropine block the fall in FEV1 but not the cough induced by hypotonic mist. *Thorax* **39**:766–70.
- Garcia-Elias A, Mrkonjic S, Pardo-Pastor C, Inada H, Hellmich UA, Rubio-Moscardó F, Plata C, Gaudet R, Vicente R, and Valverde MA (2013) Phosphatidylinositol-4,5-bisphosphate-dependent rearrangement of TRPV4 cytosolic tails enables channel activation by physiological stimuli. *Proc Natl Acad Sci* 2–7.

- Gardiner PJ, and Browne JL (1984) Tussive activity of inhaled PGD₂ in the cat and characterisation of the receptor(s) involved. *Prostaglandins Leukot Med* **14**:153–9.
- Gavva NR, Bannon AW, Hovland DN, Lehto SG, Klionsky L, Surapaneni S, Immke DC, Henley C, Arik L, Bak A, Davis J, Ernst N, Hever G, Kuang R, Shi L, Tamir R, Wang J, Wang W, Zajic G, Zhu D, Norman MH, Louis J-C, Magal E, and Treanor JJS (2007) Repeated administration of vanilloid receptor TRPV1 antagonists attenuates hyperthermia elicited by TRPV1 blockade. *J Pharmacol Exp Ther* **323**:128–37.
- Gavva NR, Treanor JJS, Garami A, Fang L, Surapaneni S, Akrami A, Alvarez F, Bak A, Darling M, Gore A, Jang GR, Kesslak JP, Ni L, Norman MH, Palluconi G, Rose MJ, Salfi M, Tan E, Romanovsky AA, Banfield C, and Davar G (2008) Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* **136**:202–10.
- Gever JR, Cockayne DA, Dillon MP, Burnstock G, and Ford APDW (2006) Pharmacology of P2X channels. *Pflugers Arch-ERJ* **452**: 513-537
- Gever JR, Soto R, Henningsen RA, Martin RS, Hackos DH, Panicker S, Rubas W, Oglesby IB, Dillon MP, Milla ME, Burnstock G, and Ford APDW (2010) AF-353, a novel, potent and orally bioavailable P2X₃/P2X_{2/3} receptor antagonist. *Br J Pharmacol* **160**:1387–98.
- Global Strategy for Asthma Management and Prevention, Global Initiative for Asthma (GINA) 2007. Available from: <http://www.ginasthma.org/>.
- Gompertz S, Bayley DL, Hill SL, and Stockley RA (2001) Relationship between airway inflammation and the frequency of exacerbations in patients with smoking related COPD. *Thorax* **56**:36–41
- Grace MS, Birrell MA, Dubuis E, Maher SA, and Belvisi MG (2012) Transient receptor potential channels mediate the tussive response to prostaglandin E₂ and bradykinin. *Thorax* **67**:891–900.
- Grace MS, and Belvisi MG (2011) TRPA1 receptors in cough. *Pulm Pharmacol Ther* **24**:286–8.
- Grace MS, Dubuis E, Birrell MA, and Belvisi MG (2013) Pre-clinical studies in cough research: role of Transient Receptor Potential (TRP) channels. *Pulm Pharmacol Ther* **26**:498–507.
- Grace MS, Lieu T, Darby B, Abogadie FC, Veldhuis N, Bunnett NW, and McIntyre P (2014) The Tyrosine Kinase Inhibitor Bafetinib Inhibits PAR₂ -induced Activation of TRPV₄ In Vitro and Pain In Vivo. *Br J Pharmacol* **61**.
- Grant AD, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, Altier C, Cenac N, Zamponi GW, Bautista-Cruz F, Lopez CB, Joseph EK, Levine JD, Liedtke W, Vanner S, Vergnolle N, Geppetti P, and Bunnett NW (2007) Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J Physiol* **578**:715–33.
- Groneberg DA, Niimi A, Dinh QT, Cosio B, Hew M, Fischer A, and Chung KF (2004) Increased expression of transient receptor potential vanilloid-1 in airway nerves of chronic cough. *Am J Respir Crit Care Med* **170**:1276–80.

- Güler AD, Lee H, Iida T, Shimizu I, Tominaga M, and Caterina M (2002) Heat-evoked activation of the ion channel, TRPV4. *J Neurosci* **22**:6408–6414.
- Gunthorpe MJ, and Chizh BA (2009) Clinical development of TRPV1 antagonists: targeting a pivotal point in the pain pathway. *Drug Discov Today* **14**:56–67.
- Haji A, Ohi Y, and Tsunekawa S (2008) N-methyl-D-aspartate mechanisms in depolarization of augmenting expiratory neurons during the expulsive phase of fictive cough in decerebrate cats. *Neuropharmacology* **54**:1120–1127.
- Hallstrand TS (2012) New insights into pathogenesis of exercise-induced bronchoconstriction. *Curr Opin Allergy Clin Immunol*. **12**:42-8
- Hamanaka K, Jian MY, Townsley MI, King JA, Liedtke W, Weber DS, Eyal FG, Clapp MC, and Parker JC (2010) TRPV4 channels augment macrophage activation and ventilator-induced lung injury. *Am J Physiol Lung Cell Mol Physiol*; **299**: L353-62.
- Hanáček J, Davies A, and Widdicombe JG (1984) Influence of lung stretch receptors on the cough reflex in rabbits. *Respiration* **45**:161–8.
- Haque RA, Usmani OS, and Barnes PJ (2005) Chronic idiopathic cough: a discrete clinical entity? *Chest* **127**:1710–1713.
- Hilton ECY, Baverel PG, Woodcock A, Van Der Graaf PH, and Smith JA (2013) Pharmacodynamic modeling of cough responses to capsaicin inhalation calls into question the utility of the C5 end point. *J Allergy Clin Immunol* **132**:847-55
- Hinman A, Chuang H-H, Bautista DM, and Julius D (2006) TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A* **103**:19564–8.
- Ho CY, Gu Q, Lin YS, and Lee LY (2001) Sensitivity of vagal afferent endings to chemical irritants in the rat lung. *Respir Physiol* **127**:113–124.
- Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, and Paré PD (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* **350**:2645–2653
- Honore P, Mikusa J, Bianchi B, McDonald H, Cartmell J, Faltynek C, and Jarvis MF (2002) TNP-ATP, a potent P2X3 receptor antagonist, blocks acetic acid-induced abdominal constriction in mice: Comparison with reference analgesics. *Pain* **96**:99–105.
- Hu Y, Liu Z, Yu X, Pasricha PJ, Undem BJ, and Yu S (2014) Increased acid responsiveness in vagal sensory neurons in a guinea pig model of eosinophilic esophagitis. *Am J Physiol Gastrointest Liver Physiol* **307**:G149–57.
- Hunter DD, and Undem BJ (1999) Identification and substance P content of vagal afferent neurons innervating the epithelium of the guinea pig trachea. *Am J Respir Crit Care Med* **159**:1943–8.
- Hunter DD, Myers AC., and Undem BJ (200) Nerve growth factor induced phenotypic switch in guinea pig airway sensory neurons. *Am J Respir Crit Care Med* **161**:1985-90

- Idzko M, Hammad H, van Nimwegen M, Kool M, Willart MAM, Muskens F, Hoogsteden HC, Luttmann W, Ferrari D, Di Virgilio F, Virchow JC, and Lambrecht BN (2007) Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nat Med* **13**:913–919.
- Irwin RS, Curley FJ, and French CL (1990) Chronic cough. The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. *Am Rev Respir Dis* **141**:640–647
- Irwin RS, and Curley FJ (1991) The treatment of cough. A comprehensive review. *Chest* **99**:1477–1484
- Irwin RS, Boulet LP, Cloutier MM, Fuller R, Gold PM, Hoffstein V, Ing AJ, McCool FD, O’Byrne P, Poe RH, Prakash UB, Pratter MR, and Rubin BK (1998) Managing cough as a defense mechanism and as a symptom. A consensus panel report of the American College of Chest Physicians. *Chest* **114**:133S–181S.
- Irwin RS (2006) Complications of cough: ACCP evidence-based clinical practice guidelines. *Chest* **129**:54S–58S
- Irwin RS, Baumann MH, Bolser DC, Boulet L-P, Braman SS, Brightling CE, Brown KK, Canning BJ, Chang AB, Diczpinigaitis P V, Eccles R, Glomb WB, Goldstein LB, Graham LM, Hargreave FE, Kvale PA, Lewis SZ, McCool FD, McCrory DC, Prakash UBS, Pratter MR, Rosen MJ, Schulman E, Shannon JJ, Smith Hammond C, Tarlo SM, and (ACCP) AC of CP (2006) Diagnosis and management of cough executive summary: ACCP evidence-based clinical practice guidelines. *Chest* **129**: 1S-23S
- Jang Y, Lee Y, Kim SM, Yang YD, Jung J, and Oh U (2012) Quantitative analysis of TRP channel genes in mouse organs. *Arch Pharm Res* **35**:1823–1830.
- Janson C, Chinn S, Jarvis D, and Burney P (2001) Determinants of cough in young adults participating in the European Community Respiratory Health Survey. *Eur Respir J* **18**:647–654
- Jaquemar D, Schenker T, and Trueb B (1999) An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *J Biol Chem* **274**:7325–33.
- Jarvis MF (2003) Contributions of P2X3 homomeric and heteromeric channels to acute and chronic pain. *Expert Opin Ther Targets* **7**:513–522.
- Jarvis MF, Wismer CT, Schweitzer E, Yu H, van Biesen T, Lynch KJ, Burgard EC, and Kowaluk EA (2001) Modulation of BzATP and formalin induced nociception: attenuation by the P2X receptor antagonist, TNP-ATP and enhancement by the P2X(3) allosteric modulator, cibacron blue. *Br J Pharmacol* **132**:259–269.
- Jia Y, McLeod RL, Wang X, Parra LE, Egan RW, and Hey JA (2002) Anandamide induces cough in conscious guinea-pigs through VR1 receptors. *Br J Pharmacol* **137**:831–836,
- Jia Y, Wang X, Varty L, Rizzo CA, Yang R, Correll CC, Phelps PT, Egan RW, and Hey JA (2004) Functional TRPV4 channels are expressed in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **287**:L272–8.

- Jiang L-H, Kim M, Spelta V, Bo X, Surprenant A, and North RA (2003) Subunit arrangement in P2X receptors. *J Neurosci* **23**:8903–8910.
- Jin Y-H, Bailey TW, Li B-Y, Schild JH, and Andresen MC (2004) Purinergic and vanilloid receptor activation releases glutamate from separate cranial afferent terminals in nucleus tractus solitarius. *J Neurosci* **24**:4709–4717.
- Joos GF, De Swert KO, Schelfhout V, and Pauwels RA (2003) The role of neural inflammation in asthma and chronic obstructive pulmonary disease. *Ann N Y Acad Sci* **992**:218–230.
- Jordt S, Bautista DM, Chuang H, Meng ID, and Julius D (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature* **427**. 260-5
- Jordt SE, Tominaga M, and Julius D (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci U S A* **97**:8134–9.
- Joris L, Dab I, and Quinton PM (1993) Elemental composition of human airway surface fluid in healthy and diseased airways. *Am Rev Respir Dis* **148**:1633–7.
- Kagaya M, Lamb J, Robbins J, Page CP, and Spina D (2002) Characterization of the anandamide induced depolarization of guinea-pig isolated vagus nerve. *Br J Pharmacol* **137**:39–48.
- Kajekar R, Proud D, Myers AC, Meeker SN, and Undem BJ (1999) Characterization of vagal afferent subtypes stimulated by bradykinin in guinea pig trachea. *J Pharmacol Exp Ther* **289**:682–687
- Kamei J, Iwamoto Y, Suzuki T, Misawa M, Nagase H, and Kasuya Y (1993) Antitussive effects of naltrindole, a selective delta-opioid receptor antagonist, in mice and rats. *Eur J Pharmacol* **249**:161–5.
- Kamei J, Takahashi Y, Yoshikawa Y, and Saitoh A (2005) Involvement of P2X receptor subtypes in ATP-induced enhancement of the cough reflex sensitivity. *Eur J Pharmacol* **528**:158–61.
- Kaneko Y, and Szallasi A (2014) Transient receptor potential (TRP) channels: a clinical perspective. *Br J Pharmacol* **171**:2474–2507.
- Kanner RE, Connett JE, Williams DE, and Buist AS (1999) Effects of randomized assignment to a smoking cessation intervention and changes in smoking habits on respiratory symptoms in smokers with early chronic obstructive pulmonary disease: the Lung Health Study. *AJM* **106**:410–416
- Karlsson JA, and Fuller RW (1999) Pharmacological regulation of the cough reflex--from experimental models to antitussive effects in Man. *Pulm Pharmacol Ther* **12**:215–28.
- Karlsson JA, Hansson L, Wollmer P, and Dahlbäck M (1991) Regional sensitivity of the respiratory tract to stimuli causing cough and reflex bronchoconstriction. *Respir Med* **85 Suppl A**:47–50.
- Keatings VM, Collins PD, Scott DM, and Barnes PJ (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* **153**:530–534

- Keatings VM, Jatakanon A, Worsdell YM, and Barnes PJ (1997) Effects of inhaled and oral glucocorticoids on inflammatory indices in asthma and COPD. *Am J Respir Crit Care Med* **155**:542–548.
- Kelsall A, Houghton LA, Jones H, Decalmer S, McGuinness K, and Smith JA (2011) A novel approach to studying the relationship between subjective and objective measures of cough. *Chest* **139**:569–75.
- Key AL, Holt K, Hamilton A, Smith JA, and Earis JE (2010) Objective cough frequency in Idiopathic Pulmonary Fibrosis. *Cough* **6**:4.
- Khalid S, Murdoch R, Newlands A, Smart K, Kelsall A, Holt K, Dockry R, Woodcock A, and Smith JA. (2014) Transient receptor potential vanilloid 1 (TRPV1) antagonism in patients with refractory chronic cough: A double-blind randomized controlled trial. *J Allergy Clin Immunol* **1**:56-62
- Kogan MD, Pappas G, Yu SM, and Kotelchuck M (1994) Over-the-counter medication use among US preschool-age children. *JAMA* **272**:1025–30.
- Kollarik M, and Undem BJ (2002) Mechanisms of acid-induced activation of airway afferent nerve fibres in guinea-pig. *J Physiol* **543**:591–600
- Koskela HO, Kontra KM, Purokivi MK, and Randell JT (2005) Interpretation of cough provoked by airway challenges. *Chest* **128**:3329–3335
- Koskela HO, Purokivi MK, Kontra KM, Taivainen AH, and Tukiainen HO (2008) Hypertonic saline cough provocation test with salbutamol pre-treatment: Evidence for sensorineural dysfunction in asthma. *Clin Exp Allergy* **38**:1100–1107.
- Krørup AL, Ny L, Astrand M, Bajor A, Hvid-Jensen F, Hansen MB, Simrén M, Funch-Jensen P, and Drewes AM (2011) Randomised clinical trial: the efficacy of a transient receptor potential vanilloid 1 antagonist AZD1386 in human oesophageal pain. *Aliment Pharmacol Ther* **33**:1113–22.
- Kubin L, Alheid GF, Zuperku EJ, and McCrimmon DR (2006) Central pathways of pulmonary and lower airway vagal afferents. *J Appl Physiol* **101**:618–627.
- Kwan KY, Allchorne AJ, Vollrath M a, Christensen AP, Zhang D-S, Woolf CJ, and Corey DP (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* **50**:277–89.
- Kwong K, Kollarik M, Nassenstein C, Ru F, and Undem BJ (2008) P2X2 receptors differentiate placodal vs . neural crest C-fiber phenotypes innervating guinea pig lungs and esophagus. **21224**:858–865.
- Kwong K, Kollarik M, Nassenstein C, Ru F, and Undem BJ (2008) P2X2 receptors differentiate placodal vs. neural crest C-fiber phenotypes innervating guinea pig lungs and esophagus. *Am J Physiol - Lung Cell Mol Physiol* **295**:L858–L865
- Laitinen LA, Heino M, Laitinen A, Kava T, and Haahtela T (1985) Damage of the airway epithelium and bronchial reactivity in patients with asthma. *Am Rev Respir Dis* **131**:599–606.

- Lalloo UG, Barnes PJ, and Chung KF (1996) Pathophysiology and clinical presentations of cough☆☆☆★. *J Allergy Clin Immunol* **98**:S91–S97.
- Lalloo UG, Fox AJ, Belvisi MG, Chung KF, and Barnes PJ (1995) Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs. *J Appl Physiol* **79**:1082–1087.
- Lang F, Busch GL, Ritter M, Völkl H, Waldegger S, Gulbins E, and Häussinger D (1998) Functional significance of cell volume regulatory mechanisms. *Physiol Rev* **78**:247–306.
- Laude EA, Higgins KS, and Morice AH (1993) A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man. *Pulm Pharmacol* **6**:171–175.
- Laude EA, Morice AH, and Grattan TJ (1994) The antitussive effects of menthol, camphor and cineole in conscious guinea-pigs. *Pulm Pharmacol* **7**:179–84.
- Lechner SG, Markworth S, Poole K, Smith ESJ, Lapatsina L, Frahm S, May M, Pischke S, Suzuki M, Ibañez-Tallon I, Luft FC, Jordan J, and Lewin GR (2011) The molecular and cellular identity of peripheral osmoreceptors. *Neuron* **69**:332–44.
- Lee M-G (2006) Effect of Nociceptin in Acid-evoked Cough and Airway Sensory Nerve Activation in Guinea Pigs. *Am J Respir Crit Care Med* **173**:271–275.
- Lemanske RF, and Busse WW (2003) 6. Asthma. *J Allergy Clin Immunol* **111**:S502–S519.
- Lewis C, Neidhart S, Holy C, North RA, Buell G, and Surprenant A (1995) Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature* **377**:432–435.
- Lewis CA, Ambrose C, Banner K, Battram C, Butler K, Giddings J, Mok J, Nasra J, Winny C, and Poll C (2007) Animal models of cough: literature review and presentation of a novel cigarette smoke-enhanced cough model in the guinea-pig. *Pulm Pharmacol Ther* **20**:325–33.
- Liedtke W, Choe Y, Martí-Renom MA, Bell AM, Denis CS, Sali A, Hudspeth AJ, Friedman JM, and Heller S (2000) Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell* **103**:525–35.
- Liedtke W, and Friedman JM (2003) Abnormal osmotic regulation in trpv4-/- mice. *Proc Natl Acad Sci U S A* **100**:13698–703.
- Lieu TM, Myers AC, Meeker S, and Undem BJ (2012) TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors. *Am J Physiol - Lung Cell Mol Physiol* **302**:L941–8
- Lin C-C, Lee I-T, Yang Y-L, Lee C-W, Kou YR, and Yang C-M (2010) Induction of COX-2/PGE2/IL-6 is crucial for cigarette smoke extract-induced airway inflammation: Role of TLR4-dependent NADPH oxidase activation. *Free Radic Biol Med* **48**:240–254,
- Løkke A, Lange P, Scharling H, Fabricius P, and Vestbo J (2006) Developing COPD: a 25 year follow up study of the general population. *Thorax* **61**:935–939

- Lommatzsch M, Cicko S, Müller T, Lucattelli M, Bratke K, Stoll P, Grimm M, Dürk T, Zissel G, Ferrari D, Di Virgilio F, Soricther S, Lungarella G, Virchow JC, and Idzko M (2010) Extracellular adenosine triphosphate and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **181**:928–934.
- Lowry RH, Wood AM, and Higenbottam TW (1988) Effects of pH and osmolarity on aerosol-induced cough in normal volunteers. *Clin Sci* **74**:373–376
- Lundberg JM, Franco-Cereceda A, Hua X, Hökfelt T, and Fischer JA (1985) Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsaicin. *Eur J Pharmacol* **108**:315–9.
- Macpherson LJ, Geierstanger BH, Viswanath V, Bandell M, Eid SR, Hwang S, and Patapoutian A (2005) The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. *Curr Biol* **15**:929–34.
- Madison JM, and Irwin RS (2010) Cough: a worldwide problem. *Otolaryngol Clin North Am* **43**:1–13, vii.
- Maher SA, and Belvisi MG (2010) Prostanoids and the cough reflex. *Lung* **188 Suppl** :S9–12.
- Maher SA, Birrell MA, and Belvisi MG (2009) Prostaglandin E2 mediates cough via the EP3 receptor: implications for future disease therapy. *Am J Respir Crit Care Med* **180**:923–928.
- Mazzone SB (2004) Sensory regulation of the cough reflex. *Pulm Pharmacol Ther* **17**:361–368
- Mazzone SB, and Canning BJ (2002) Central nervous system control of the airways: Pharmacological implications. *Curr Opin Pharmacol* **2**:220–228.
- McAlexander MA, Luttmann MA, Hunsberger GE, and Undem BJ (2014) Transient Receptor Potential Vanilloid 4 (TRPV4) activation constricts the human bronchus via the release of cysteinyl leukotrienes. *J Pharmacol Exp Ther* **4**:1–25.
- McCluskie K, Birrell MA, Wong S, and Belvisi MG (2004) Nitric oxide as a noninvasive biomarker of lipopolysaccharide-induced airway inflammation: possible role in lung neutrophilia. *J Pharmacol Exp Ther* **311**:625–633
- McGarvey L, Polley L, Yaman N, Heaney L, Cardwell C, Murtagh E, Ramsey J, Macmahon J, and Costello RW (2008) Impact of cough across different chronic respiratory diseases: comparison of two cough-specific health-related quality of life questionnaires. *Chest* **134**:295–302.
- McGarvey LP, and Morice a H (2006) Clinical cough and its mechanisms. *Respir Physiol Neurobiol* **152**:363–71.
- McGarvey LP, Heaney LG, Lawson JT, Johnston BT, Scally CM, Ennis M, Shepherd DR, and MacMahon J (1998) Evaluation and outcome of patients with chronic non-productive cough using a comprehensive diagnostic protocol. *Thorax* **53**:738–743, Department of Respiratory Medicine, Belfast City Hospital, UK.

- McGarvey LPA, Forsythe P, Heaney LG, MacMahoir J, and Ennis M (1999) Bronchoalveolar lavage findings in patients with chronic nonproductive cough. *Eur Respir J* **13**:59–65.
- McGarvey LP, Butler CA., Stokesberry S, Polley L, McQuaid S, Abdullah H, Ashraf S, McGahon MK, Curtis TM, Arron J, Choy D, Warke TJ, Bradding P, Ennis M, Zholos A, Costello RW, and Heaney LG (2013) Increased expression of bronchial epithelial transient receptor potential vanilloid 1 channels in patients with severe asthma. *J Allergy Clin Immunol*, **133**:704–12
- McGuinness K, Holt K, Dockry R, and Smith J (2012) P159 Validation of the VitaloJAK 24 Hour Ambulatory Cough Monitor. *Thorax* **67**:A131–A131.
- McGuinness K, Morice A, Woodcock A, and Smith J (2008) The Leicester Cough Monitor: a semi-automated, semi-validated cough detection system? *Eur Respir J* **32**:529–30; author reply 530–1.
- McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM, and Fanger CM (2007) TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci* **104**:13525–13530.
- Millqvist E (2011) The airway sensory hyperreactivity syndrome. *Pulm Pharmacol Ther* **24**:263–266
- Mizuno A, Matsumoto N, Imai M, and Suzuki M (2003) Impaired osmotic sensation in mice lacking TRPV4. *Am J Physiol Cell Physiol* **285**:C96–101.
- Mochizuki T, Sokabe T, Araki I, Fujishita K, Shibasaki K, Uchida K, Naruse K, Koizumi S, Takeda M, and Tominaga M (2009) The TRPV4 cation channel mediates stretch-evoked Ca²⁺ influx and ATP release in primary urothelial cell cultures. *J Biol Chem* **284**:21257–21264.
- Mohsenin A, and Blackburn MR (2006) Adenosine signaling in asthma and chronic obstructive pulmonary disease. *Curr Opin Pulm Med* **12**:54–59.
- Montell C, and Rubin GM (1989) Molecular characterization of the *Drosophila* trp locus: a putative integral membrane protein required for phototransduction. *Neuron* **2**:1313–23.
- Montuschi P, Kharitonov SA, Ciabattini G, and Barnes PJ (2003) Exhaled leukotrienes and prostaglandins in COPD. *Thorax* **58**:585–588
- Moran MM, McAlexander MA, Bíró T, and Szallasi A (2011) Transient receptor potential channels as therapeutic targets. *Nat Rev Drug Discov* **10**:601–20.
- Morice AH, Kastelik JA, and Thompson R (2001) Cough challenge in the assessment of cough reflex. *Br J Clin Pharmacol* **52**:365–375
- Morice AH (2004) The diagnosis and management of chronic cough. *Eur Respir J* **24**:481–492
- Morice AH, McGarvey L, and Pavord I (2006) Recommendations for the management of cough in adults. *Thorax* **61 Suppl 1**:i1–i24.

- Morice AH, Fontana GA, Belvisi MG, Birring SS, Chung KF, Diczpinigaitis P V, Kastelik JA, McGarvey LP, Smith JA, Tatar M, and Widdicombe J (2007) ERS guidelines on the assessment of cough. *Eur Respir J* **29**:1256–1276
- Morice AH, Menon MS, Mulrennan SA, Everett CF, Wright C, Jackson J, and Thompson R (2007) Opiate therapy in chronic cough. *Am J Respir Crit Care Med* **175**:312–5.
- Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, Tominaga T, Narumiya S, and Tominaga M (2005) Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain* **1**:3
- Mortaz E, Folkerts G, Nijkamp FP, and Henricks PAJ (2010) ATP and the pathogenesis of COPD. *Eur J Pharmacol* **638**:1–4.
- Mortola J, Sant'Ambrogio G, and Clement MG (1975) Localization of irritant receptors in the airways of the dog. *Respir Physiol* **24**:107–114.
- Mukhopadhyay I, Kulkarni A, Aranake S, Karnik P, Shetty M, Thorat S, Ghosh I, Wale D, Bhosale V, and Khairatkar-Joshi N (2014) Transient Receptor Potential Ankyrin 1 Receptor Activation In Vitro and In Vivo by Pro-tussive Agents: GRC 17536 as a Promising Anti-Tussive Therapeutic. *PLoS One* **9**:e97005–e97005.
- Mund E, Christensson B, Grönneberg R, and Larsson K (2005) Noneosinophilic CD4 lymphocytic airway inflammation in menopausal women with chronic dry cough. *Chest* **127**:1714–1721.
- Mutolo D, Bongianini F, Cinelli E, and Pantaleo T (2010) Depression of cough reflex by microinjections of antitussive agents into caudal ventral respiratory group of the rabbit. *J Appl Physiol* **109**:1002–1010.
- Mutolo D, Bongianini F, Fontana GA, and Pantaleo T (2007) The role of excitatory amino acids and substance P in the mediation of the cough reflex within the nucleus tractus solitarius of the rabbit. *Brain Res Bull* **74**:284–293.
- Myers AC, Kajekar R, and Undem BJ (2002) Allergic inflammation-induced neuropeptide production in rapidly adapting afferent nerves in guinea pig airways. *Am J Physiol Lung Cell Mol Physiol* **282**:L775–81.
- Naruyima S, Sugimoto Y and Ushikubi F (1999) Prostanoid receptors: structures, properties and functions. *Physiol Rev* **79**: 1193-1226
- Nasra J, and Belvisi MG (2009) Modulation of sensory nerve function and the cough reflex: Understanding disease pathogenesis. *Pharmacol Ther* **124**:354–375
- Nassenstein C, Kwong K, Taylor-Clark T, Kollarik M, Macglashan DM, Braun A, and Undem BJ (2008) Expression and function of the ion channel TRPA1 in vagal afferent nerves innervating mouse lungs. *J Physiol* **586**:1595–1604
- Nassini R, Materazzi S, de Siena G, de Cesaris F, and Geppetti P (2010) Transient receptor potential channels as novel drug targets in respiratory diseases. *Curr Opin Investig Drugs* **11**:535–542.

- Ni D, Gu Q, Hu H-Z, Gao N, Zhu MX, and Lee L-Y (2006) Thermal sensitivity of isolated vagal pulmonary sensory neurons: role of transient receptor potential vanilloid receptors. *Am J Physiol Regul Integr Comp Physiol* **291**:R541–50.
- Nieto L, de Diego A, Perpiñá M, Compte L, Garrigues V, Martínez E, and Ponce J (2003) Cough reflex testing with inhaled capsaicin in the study of chronic cough. *Respir Med* **97**:393–400.
- Niimi A (2011) Cough and Asthma. *Curr Respir Med Rev* **7**:47–54.
- Nilius B, Prenen J, Wissenbach U, Bödding M, and Droogmans G (2001) Differential activation of the volume-sensitive cation channel TRP12 (OTRPC4) and volume-regulated anion currents in HEK-293 cells. *Pflugers Arch* **443**:227–33.
- Nilius B, and Szallasi A (2014) Transient Receptor Potential Channels as Drug Targets: From the Science of Basic Research to the Art of Medicine. *Pharmacol Rev* **66**:676–814.
- North RA, and Jarvis MF (2013) P2X receptors as drug targets. *Mol Pharmacol* **83**:759–69.
- North RA, and Surprenant A (2000) Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol* **40**:563–580.
- Osman LM, McKenzie L, Cairns J, Friend JA, Godden DJ, Legge JS, and Douglas JG (2001) Patient weighting of importance of asthma symptoms. *Thorax* **56**:138–142.
- Pacheco A (2014) Chronic cough: from a complex dysfunction of the neurological circuit to the production of persistent cough. *Thorax* **69**:881–3.
- Patel HJ, Birrell MA, Crispino N, Hele DJ, Venkatesan P, Barnes PJ, Yacoub MH, and Belvisi MG (2003) Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in guinea-pigs by cannabinoid (CB2) receptor activation. *Br J Pharmacol* **140**:261–8.
- Patel HJ, Birrell MA, Crispino N, Hele DJ, Venkatesan P, Barnes PJ, Yacoub MH, and Belvisi MG (2003b) Inhibition of guinea-pig and human sensory nerve activity and the cough reflex in guinea-pigs by cannabinoid (CB2) receptor activation. *Br J Pharmacol* **140**:261–268, Respiratory Pharmacology Group, Guy Scadding Building, The National Heart & Lung Institute, Faculty of Medicine, Imperial College London, Dovehouse Street, London SW3 6LY.
- Pavord ID (2004) Cough and asthma. *Pulm Pharmacol Ther* **17**:399–402, Elsevier Ltd.
- Pedersen KE, Meeker SN, Riccio MM, and Undem BJ (1998) Selective stimulation of jugular ganglion afferent neurons in guinea pig airways by hypertonic saline. *J Appl Physiol* **84**:499–506.
- Pelleg A, and Hurt CM (1996) Mechanism of action of ATP on canine pulmonary vagal C fibre nerve terminals. *J Physiol* **490**:265–275.
- Pelleg A, and Schulman ES (2002) Adenosine 5'-triphosphate axis in obstructive airway diseases. *Am J Ther* **9**:454–464.
- Pellegrino R, Wilson O, Jenouri G, and Rodarte JR (1996) Lung mechanics during induced bronchoconstriction. *J Appl Physiol* **81**:964–75

- Picazo-Juárez G, Romero-Suárez S, Nieto-Posadas A, Llorente I, Jara-Oseguera A, Briggs M, McIntosh TJ, Simon SA, Ladrón-de-Guevara E, Islas LD, and Rosenbaum T (2011) Identification of a binding motif in the S5 helix that confers cholesterol sensitivity to the TRPV1 ion channel. *J Biol Chem* **286**:24966–76.
- Pisarri TE, Jonzon A, Coleridge HM, and Coleridge JC (1992) Vagal afferent and reflex responses to changes in surface osmolarity in lower airways of dogs. *J Appl Physiol* **73**:2305–2313.
- Plant TD, and Strotmann R (2007) TRPV4: A Multifunctional Nonselective Cation Channel with Complex Regulation. In: Liedtke WB, Heller S, editors. *TRP Ion Channel Function in Sensory Transduction and Cellular Signaling Cascades*. Boca Raton (FL): CRC Press; 2007. Chapter 9. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK5242/>
- Polley L, Yaman N, Heaney L, Cardwell C, Murtagh E, Ramsey J, Macmahon J, Costello RW, and McGarvey L (2008) Impact of cough across different chronic respiratory diseases: comparison of two cough-specific health-related quality of life questionnaires. *Chest* **134**:295–302.
- Polosa R, and Holgate ST (2006) Adenosine receptors as promising therapeutic targets for drug development in chronic airway inflammation. *Curr Drug Targets* **7**:699–706.
- Poole DP, Amadesi S, Veldhuis NA, Abogadie FC, Lieu T, Darby W, Liedtke W, Lew MJ, McIntyre P, and Bunnett NW (2013) Protease-activated receptor 2 (PAR2) protein and transient receptor potential vanilloid 4 (TRPV4) protein coupling is required for sustained inflammatory signaling. *J Biol Chem* **288**:5790–802.
- Pratter MR (2006) Cough and the common cold: ACCP evidence-based clinical practice guidelines. *Chest* **129**:220S–221S, American College of Chest Physicians
- Preti D, Szallasi A, and Patacchini R (2012) TRP channels as therapeutic targets in airway disorders: a patent review. *Expert Opin Ther Pat* **22**:663–695, University of Ferrara, Department of Pharmaceutical Sciences , via Fossato di Mortara 17/19, 44121 , Italy.
- Prudon B, Birring SS, Vara DD, Hall AP, Thompson JP, and Pavord ID (2005) Cough and glottic-stop reflex sensitivity in health and disease. *Chest* **127**:550–7.
- Rahaman SO, Grove LM, Paruchuri S, Southern BD, Abraham S, Niese KA, Scheraga RG, Ghosh S, Thodeti CK, Zhang DX, Moran MM, Schilling WP, Tschumperlin DJ, and Olman MA (2014) TRPV4 mediates myofibroblast differentiation and pulmonary fibrosis in mice. *J Clin Invest* **124**:5225–38.
- Eng SR, Dykes IM, Lanier J, Fedtsova N and Turner E (2007) POU-domain factor Brn3a regulates both distinct and common programs of gene expression in the spinal and trigeminal sensory ganglia. *Neural Development* **2**: 3
- Ramsey IS, Delling M, and Clapham DE (2006) An introduction to TRP channels. *Annu Rev Physiol* **68**:619–47.
- Rennard S, Decramer M, Calverley PMA, Pride NB, Soriano JB, Vermeire PA, and Vestbo J (2002) Impact of COPD in North America and Europe in 2000: subjects' perspective of Confronting COPD International Survey. *Eur Respir J* **20**:799–805.

- Riccio M, Kummer W, Biglari B, Myers AC, and Undem BJ (1996) Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways. *J Physiol* **496**:521–530
- Riccio M, Myers A, and Undem B (1996) Immunomodulation of afferent neurons in guinea-pig isolated airway. *J Physiol* **491**:499–509.
- Ricco MM, Kummer W, Biglari B, Myers AC, and Undem BJ (1996) Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways. *J Physiol* **496** (Pt 2):521–30.
- Rolin S, Masereel B and Dogne JM (2006) Prostanoids as pharmacological targets in COPD and asthma. *Eur J Pharmacol* **533**: 89-100
- Round P, Priestley A, and Robinson J (2011) An investigation of the safety and pharmacokinetics of the novel TRPV1 antagonist XEN-D0501 in healthy subjects. *Br J Clin Pharmacol* **72**:921–931.
- Sant'Ambrogio G, Remmers JE, de Groot WJ, Callas G, and Mortola JP (1978) Localization of rapidly adapting receptors in the trachea and main stem bronchus of the dog. *Respir Physiol* **33**:359–366.
- Sant'Ambrogio G, and Widdicombe J (2001) Reflexes from airway rapidly adapting receptors. *Respir Physiol* **125**:33-45
- Sato D, Sato T, Urata Y, Okajima T, Kawamura S, Kurita M, Takahashi K, Nanno M, Watahiki A, Kokubun S, Shimizu Y, Kasahara E, Shoji N, Sasano T, and Ichikawa H (2014) Distribution of TRPVs, P2X3, and parvalbumin in the human nodose ganglion. *Cell Mol Neurobiol* **34**:851–858.
- Sawada Y, Hosokawa H, Matsumura K, and Kobayashi S (2008) Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. *Eur J Neurosci* **27**:1131–42.
- Schappert SM, and Burt CW (2006) Ambulatory care visits to physician offices, hospital outpatient departments, and emergency departments: United States, 2001-02. *Vital Health Stat* **13** 1–66
- Schappert SM, and Rechtsteiner EA (2011) Ambulatory medical care utilization estimates for 2007. *Vital Health Stat* **13** 1–38, Division of Health Statistics, Center for Disease Control and Prevention, National Center for Health Statistics, Hyattsville, MD 20782, USA.
- Schelegle ES, and Green JF (2001) An overview of the anatomy and physiology of slowly adapting pulmonary stretch receptors. *Respir Physiol* **125**:17–31
- Schelegle ES (2003) Functional morphology and physiology of slowly adapting pulmonary stretch receptors. *Anat Rec A Discov Mol Cell Evol Biol* **270**:11–6.
- Schroeder K, and Fahey T (2002) Primary care the counter cough medicines for acute cough in adults. **324**:1–6.
- Seminario-Vidal L, Okada SF, Sesma JI, Kreda SM, van Heusden CA, Zhu Y, Jones LC, O'Neal WK, Penuela S, Laird DW, Boucher RC, and Lazarowski ER (2011) Rho signaling regulates pannexin 1-mediated ATP release from airway epithelia. *J Biol Chem* **286**:26277–86.

- Sesoko S, and Kaneko Y (1985) Cough associated with the use of captopril. *Arch Intern Med* **145**:1524.
- Sethi JM, and Rochester CL (2000) Smoking and chronic obstructive pulmonary disease. *Clin Chest Med* **21**:67–86.
- Sharif Naeini R, Witty M-F, Séguéla P, and Bourque CW (2006) An N-terminal variant of Trpv1 channel is required for osmosensory transduction. *Nat Neurosci* **9**:93–98.
- Shields MD, Bush A, Everard ML, McKenzie S, and Primhak R (2008) BTS guidelines: Recommendations for the assessment and management of cough in children. *Thorax* **63 Suppl 3**:iii1–iii15.
- Simpson G (1999) Investigation and management of persistent dry cough. *Thorax* **54**: 469-70
- Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, and Davis JB (2000) The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol* **129**:227–230
- Smit LAM, Kogevinas M, Antó JM, Bouzigon E, González JR, Le Moual N, Kromhout H, Carsin A-E, Pin I, Jarvis D, Vermeulen R, Janson C, Heinrich J, Gut I, Lathrop M, Valverde M a, Demenais F, and Kauffmann F (2012) Transient receptor potential genes, smoking, occupational exposures and cough in adults. *Respir Res* **13**:26.
- Smith J, Owen E, Earis J, and Woodcock A (2006) Effect of codeine on objective measurement of cough in chronic obstructive pulmonary disease. *J Allergy Clin Immunol* **117**:831–5.
- Smith J, and Woodcock A (2006) Cough and its importance in COPD. *Int J Chron Obstruct Pulmon Dis* **1**:305–314.
- Smith J, and Woodcock A (2008) New developments in the objective assessment of cough. *Lung* **186 Suppl** :S48–54
- Smith J (2010) Monitoring chronic cough: current and future techniques. *Expert Rev Respir Med* **4**:673–683
- Smith JA (2010) Assessing Efficacy of Therapy for Cough. *Otolaryngol Clin North Am* **43**:157–66
- Smith JA, Hilton ECY, Saulsberry L, and Canning BJ (2012) Antitussive effects of memantine in guinea pigs. *Chest* **141**:996–1002.
- Standring S (2005) *Gray's anatomy: the anatomical basis of clinical practice*, 39th Editi, Churchill Livingstone.
- Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, and Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* **112**:819–29.

- Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, and Plant TD (2000) OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat Cell Biol* **2**:695–702.
- Sumner H, Woodcock A, Kolsum U, Dockry R, Lazaar AL, Singh D, Vestbo J, and Smith JA (2013) Predictors of Objective Cough Frequency in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 1–32.
- Sumner H, Woodcock A, Kolsum U, Dockry R, Lazaar AL, Singh D, Vestbo J, and Smith JA (2013) Predictors of objective cough frequency in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **187**:943–9.
- Suzuki M, Mizuno A, Kodaira K, and Imai M (2003) Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem* **278**:22664–8.
- Szallasi A, and Blumberg PM (1990) Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res* **524**:106–11.
- Szallasi A, and Blumberg PM (1999) Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol Rev* **51**:159–212.
- Takahashi N, and Mori Y (2011) TRP Channels as Sensors and Signal Integrators of Redox Status Changes. *Front Pharmacol* **2**:58.
- Tatar M, Sant'Ambrogio G, and Sant'Ambrogio FB (1994) Laryngeal and tracheobronchial cough in anesthetized dogs. *J Appl Physiol* **76**:2672–2679.
- Taylor-Clark TE, McAlexander MA, Nassenstein C, Sheardown SA, Wilson S, Thornton J, Carr MJ, and Undem BJ (2008) Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononenal. *J Physiol* **586**:3447–3459.
- Ternesten-Hasséus E, Johansson K, Löwhagen O, and Millqvist E (2006) Inhalation method determines outcome of capsaicin inhalation in patients with chronic cough due to sensory hyperreactivity. *Pulm Pharmacol Ther* **19**:172–8.
- Ternesten-Hasséus E, Larsson C, Larsson S, and Millqvist E (2013) Capsaicin sensitivity in patients with chronic cough- results from a cross-sectional study. *Cough* **9**:5.
- Thorneloe KS, Sulpizio AC, Lin Z, Figueroa DJ, Clouse AK, McCafferty GP, Chendrimada TP, Lashinger ESR, Gordon E, Evans L, Misajet BA, Demarini DJ, Nation JH, Casillas LN, Marquis RW, Votta BJ, Sheardown SA, Xu X, Brooks DP, Laping NJ, and Westfall TD (2008) N-((1S)-1-[[4-((2S)-2-[[[(2,4-dichlorophenyl)sulfonyl]amino]-3-hydroxypropanoyl]-1-piperazinyl]carbonyl]-3-methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A), a novel and potent transient receptor potential vanilloid 4 channel agonist induces urinary bladder contraction and hyperactivity: Part 1. *J Pharmacol Exp Ther* **326**:432–42.
- Thorneloe KS, Cheung M, Bao W, Alsaïd H, Lenhard S, Jian M-Y, Costell M, Maniscalco-Hauk K, Krawiec J a, Olzinski A, Gordon E, Lozinskaya I, Elefante L, Qin P, Matasic DS, James C, Tunstead J, Donovan B, Kallal L, Waszkiewicz A, Vaidya K, Davenport E a, Larkin J, Burgert M, Casillas LN, Marquis RW, Ye G, Eidam HS, Goodman KB, Toomey JR, Roethke TJ, Jucker BM, Schnackenberg CG, Townsley MI, Lepore JJ, and Willette RN

- (2012) An orally active TRPV4 channel blocker prevents and resolves pulmonary edema induced by heart failure. *Sci Transl Med* **4**:159ra148.
- Tominaga M (2008) [Capsaicin receptor TRPV1]. *Brain Nerve* **60**:493–501.
- Tominaga M, and Tominaga T (2005) Structure and function of TRPV1. *Pflugers Arch* **451**: 143–50.
- Trevisani M, Siemens J, Materazzi S, Bautista DM, Nassini R, Campi B, Imamachi N, Andre E, Patacchini R, Cottrell GS, Gatti R, Basbaum AI, Bunnett NW, Julius D, and Geppetti P (2007) 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proc Natl Acad Sci* **104**:13519–13524.
- Ueno S, Moriyama T, Honda K, Kamiya HO, Sakurada T, and Katsuragi T (2003) Involvement of P2X2 and P2X3 receptors in neuropathic pain in a mouse model of chronic constriction injury. *Drug Development Research* p104–111.
- Undem BJ (2002a) Characterization of the Vanilloid Receptor 1 Antagonist Iodo-Resiniferatoxin on the Afferent and Efferent Function of Vagal Sensory C-Fibers. *J Pharmacol Exp Ther* **303**:716–722
- Undem BJ (2002b) Effect of Extracellular Calcium on Excitability of Guinea Pig Airway Vagal Afferent Nerves. *J Neurophysiol* **89**:1196–1204, Johns Hopkins School of Medicine, Department of Medicine, Baltimore, MD 21224, USA. Bundem@jhmi.edu.
- Undem BJ (2004) Subtypes of vagal afferent C-fibres in guinea-pig lungs. *J Physiol* **556**:905–917
- Undem BJ, Chuaychoo B, Lee M-G, Weinreich D, Myers AC, and Kollarik M (2004) Subtypes of vagal afferent C-fibres in guinea-pig lungs. *J Physiol* **556**:905–17.
- Undem BJ, and Nassenstein C (2009) Airway nerves and dyspnea associated with inflammatory airway disease. *Respir Physiol Neurobiol* **167**:36–44.
- Usmani OS, Belvisi MG, Patel HJ, Crispino N, Birrell MA, Korbonits M, Korbonits D, and Barnes PJ (2005) Theobromine inhibits sensory nerve activation and cough. *FASEB J* **19**:231–3.
- Vassilev ZP, Chu AF, Ruck B, Adams EH, and Marcus SM (2009) Adverse reactions to over-the-counter cough and cold products among children: the cases managed out of hospitals. *J Clin Pharm Ther* **34**:313–8.
- Veldhuis NA, Poole DP, Grace M, McIntyre P, and Bunnett NW (2015) The G Protein – Coupled Receptor – Transient Receptor Potential Channel Axis: Molecular Insights for Targeting Disorders of Sensation and Inflammation. *Pharmacol Rev* **67**:36–73.
- Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, Barnes PJ, Fabbri LM, Martinez FJ, Nishimura M, Stockley R a, Sin DD, and Rodriguez-Roisin R (2013) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* **187**:347–365.

- Virginio C, Robertson G, Surprenant A, and North RA (1998) Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X1, P2X3, and heteromeric P2X2/3 receptors. *Mol Pharmacol* **53**:969–73.
- Voets T, Talavera K, Owsianik G, and Nilius B (2005) Sensing with TRP channels. *Nat Chem Biol* **1**:85–92.
- Volpi G, Facchinetti F, Moretto N, Civelli M, and Patacchini R (2011) Cigarette smoke and α,β -unsaturated aldehydes elicit VEGF release through the p38 MAPK pathway in human airway smooth muscle cells and lung fibroblasts. *Br J Pharmacol* **163**:649–61.
- Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T, Morisseau C, Hammock BD, Fleming I, Busse R, and Nilius B (2005) Modulation of the Ca²⁺ permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res* **97**:908–15.
- Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T, Morisseau C, Hammock BD, Fleming I, Busse R, and Nilius B (2005) Modulation of the Ca²⁺ permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res* **97**:908–915.
- Vriens J, Watanabe H, Janssens a, Droogmans G, Voets T, and Nilius B (2004) Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci U S A* **101**:396–401.
- Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, and Elde R (1996) Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. *Proc Natl Acad Sci U S A* **93**:8063–8067.
- Wang ECY, Lee JM, Ruiz WG, Balestreire EM, Von Bodungen M, Barrick S, Cockayne DA, Birder LA, and Apodaca G (2005) ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest* **115**:2412–2422.
- Wareham K, Vial C, Wykes RCE, Bradding P, and Seward EP (2009) Functional evidence for the expression of P2X1, P2X4 and P2X7 receptors in human lung mast cells. *Br J Pharmacol* **157**:1215–1224.
- Watanabe H, Davis JB, Smart D, Jerman JC, Smith GD, Hayes P, Vriens J, Cairns W, Wissenbach U, Prenen J, Flockerzi V, Droogmans G, Benham CD, and Nilius B (2002) Activation of TRPV4 channels (hVRL-2/mTRP12) by phorbol derivatives. *J Biol Chem* **277**:13569–77.
- Watanabe Y, Minoguchi K, and Ohnishi T (2002) Role of prostaglandin receptor EP1 in the spinal dorsal horn in carrageenan-induced inflammatory pain. *Anesthesiology* **97**:1254–1262.
- Watanabe H, Vriens J, Prenen J, and Droogmans G (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. **424**:4–8.
- Weigand LA, Ford AP, and Undem BJ (2012) A role for ATP in bronchoconstriction-induced activation of guinea pig vagal intrapulmonary C-fibres. *J Physiol* **590**:4109–20.

- Weiler JM, Anderson SD, Randolph C, Bonini S, Craig TJ, Pearlman DS, Rundell KW, Silvers WS, Storms WW, Bernstein DI, Blessing-Moore J, Cox L, Khan DA, Lang DM, Nicklas RA, Oppenheimer J, Portnoy JM, Schuller DE, Spector SL, Tilles SA, Wallace D, Henderson W, Schwartz L, Kaufman D, Nsouli T, Shieken L, and Rosario N (2010) Pathogenesis, prevalence, diagnosis, and management of exercise-induced bronchoconstriction: A practice parameter. **105**: S1-47
- White SR, Garland A, Gitter B, Rodger I, Alger LE, Necheles J, Nawrocki AR, and Solway J (1995) Proliferation of guinea pig tracheal epithelial cells in coculture with rat dorsal root ganglion neural cells. *Am J Physiol* **268**:L957–L965.
- Widdicombe JG (1995) Neurophysiology of the cough reflex. *Eur Respir J* **8**:1193–1202,
- Widdicombe JG, and Undem BJ (2002) Summary: Central Nervous Pharmacology of Cough. *Pulm Pharmacol Ther* **15**:251–252
- Widdicombe J (2003) Functional morphology and physiology of pulmonary rapidly adapting receptors (RARs). *Anat Rec A Discov Mol Cell Evol Biol* **270**:2–10
- Willette RN, Bao W, Nerurkar S, Yue T, Doe CP, Stankus G, Turner GH, Ju H, Thomas H, Fishman CE, Sulpizio A, Behm DJ, Hoffman S, Lin Z, Lozinskaya I, Casillas LN, Lin M, Trout REL, Votta BJ, Thorneloe K, Lashinger ESR, Figueroa DJ, Marquis R, and Xu X (2008) Systemic Activation of the Transient Receptor Potential Vanilloid Subtype 4 Channel Causes Endothelial Failure and Circulatory Collapse : Part 2. *J Pharmacol Exp Ther* **326**:443–452.
- Wong CH, Matai R, and Morice AH (1999) Cough induced by low pH. *Respir Med* **93**:58–61,
- Wong S, Belvisi MG, and Birrell MA (2009) MMP/TIMP expression profiles in distinct lung disease models: implications for possible future therapies. *Respir Res* **10**:72.
- Wortley MA, Birrell MA, Maher SA, Round P, Ford J, and Belvisi MG (2014) Profiling Of Xen-D0501, A Novel Trpv1 Antagonist, In Pre-Clinical Models Of Cough. *AJRCCM* A4979.
- Wortley MA, Grace MS, Dubuis ED, Maher SA, Khalid S, Smith JA, Birrell MA, and Belvisi MG (2011) Cough And Vagal Sensory Afferent Responses To PGE2 Are Altered In A Guinea Pig Cigarette Smoke Exposure Model And In Human Smokers And COPD Patients Compared To Normal Volunteers. *Br J Pharmacol PA2 online* P064.
- Yang X-R (2006) Functional expression of transient receptor potential melastatin- and vanilloid-related channels in pulmonary arterial and aortic smooth muscle. *AJP Lung Cell Mol Physiol* **290**:L1267–L1276.
- Yousaf N, Monteiro W, Matos S, Birring SS, and Pavord ID (2013) Cough frequency in health and disease. *Eur Respir J* **41**:241–3.
- Yu S (2005) Vagal afferent nerves with nociceptive properties in guinea-pig oesophagus. *J Physiol* **563**:831–842
- Zhou Y, Sun B, Li Q, Luo P, Dong L, and Rong W (2011) Sensitivity of bronchopulmonary receptors to cold and heat mediated by transient receptor potential cation channel subtypes in an ex vivo rat lung preparation. *Respir Physiol Neurobiol* **177**:327–32.

- Zhu G, Gulsvik A, Bakke P, Ghatta S, Anderson W, Lomas D a, Silverman EK, and Pillai SG (2009) Association of TRPV4 gene polymorphisms with chronic obstructive pulmonary disease. *Hum Mol Genet* **18**:2053–62.
- Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sjørgård M, Di Marzo V, Julius D, and Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**:452–457

Appendix

The table below outlines the chemicals used throughout this thesis. Vehicles/Diluents have been added where applicable.

Drug	Source	Vehicle/Diluent (where applicable)
$\alpha\beta$ -Methylene ATP	Sigma-Aldrich	Distilled H ₂ O
Acrolein	Sigma-Aldrich	0.1% DMSO in Krebs
AF-353	Afferent Pharmaceuticals	0.1% DMSO in Krebs (<i>in vitro</i>) 10% Polyethylene Glycol in 0.9% saline (<i>in vivo</i>)
CaCl ₂	VWR	-
Capsaicin	Sigma-Aldrich	0.1% DMSO in Krebs
Citric Acid	Sigma-Aldrich	0.9% saline
Collagenase	Worthington	Ca ²⁺ -free, Mg ²⁺ -free Hank's balanced salt solution
Dil (DiIC18(3), 1,1'- dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)	Invitrogen	2% ethanol in 0.9% saline
Dispase II	Roche	Ca ²⁺ -free, Mg ²⁺ -free Hank's balanced salt solution
Ethanol	VWR	-
F12	Invitrogen	-
Fluo-4-AM	Invitrogen	Extracellular Solution
GSK1016790a	Sigma-Aldrich	0.1% DMSO in Krebs (<i>in vitro</i>) 0.1% DMSO in 0.9% saline (<i>in vivo</i>)
GSK2193874	Almirall	0.1% DMSO in Krebs (<i>in vitro</i>) 6% Cavitron 2Hydroxypropyl- β -cyclodextrin in 0.9% saline (<i>in vivo</i>)
HC030031	ChemBridge	0.1% DMSO in Krebs
HC067047	Sigma-Aldrich	0.1% DMSO in Krebs (<i>in vitro</i>)

		0.1% DMSO in 0.9% saline (<i>in vivo</i>)
HEPES	Sigma-Aldrich	-
Hyperladder IV	Bioline	-
JNJ17203212	Sigma-Aldrich	0.1% DMSO in Krebs
KCl	VWR	-
KH ₂ PO ₄	VWR	-
L15	Sigma-Aldrich	-
MgSO ₄	VWR	-
NaCl	VWR	-
NaH ₂ PO ₄	VWR	-
NaHCO ₃	VWR	-
Nuclease free water	Promega	-
Papain	Sigma-Aldrich	Hanks Balanced Salt Solution
PBS	Sigma-Aldrich	-
Percoll	Sigma-Aldrich	-
Petroleum Jelly	Vaseline	-
PGD ₂	Sigma-Aldrich	0.1% Ethanol in Krebs
PGE ₂	Sigma-Aldrich	0.1% Ethanol in Krebs
Safeview	NBS Biologicals Ltd	-
TNP-ATP	Tocris	-
Tween80	Sigma-Aldrich	-
4-alpha-phorbol-12,13-didecanoate (4αPDD)	Tocris	0.1% DMSO in Krebs