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VALIDATION AND COMPARISON OF OZONE-INDUCED HYPERTUSSIVE RESPONSES IN THE RABBIT AND GUINEA-PIG

Clay, Emlyn Robert

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University of London

KING'S COLLEGE LONDON

PHD THESIS

VALIDATION AND COMPARISON OF OZONE-INDUCED HYPERTUSSIVE
RESPONSES IN THE RABBIT AND GUINEA-PIG

Author:
EMLYN CLAY

Supervisors
PROFESSOR CLIVE PAGE
AND DR. DOMENICO
SPINA

April 14, 2015

Author's declaration

I declare that this thesis has been composed entirely by myself and the work contained herein to have principally been conducted by myself.

Acknowledgments

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Abstract

The thesis investigates establishing a hypertussive model of cough primarily in the rabbit and with comparative experiments conducted in the guinea pig. These models were then used to investigate the effectiveness of various antitussives such as codeine and levodropropizine, as well as, putative antitussives such as anticholinergics, PDE inhibitors, bronchodilators and drugs affecting targets on sensory nerves. “Hypertussive” is a poorly recognised term and it is defined in the context of this thesis to describe an inappropriately frequent and/or loud cough response when compared to normative cough responses for the same given dose and route of a given tussive stimuli.

A novel model of hypertussive cough responses was established and validated in the rabbit and guinea pig using ozone as a sensitising agent. Primary measures include cough frequency, cough magnitude, time to first cough and cough duration. In further experiments lung function parameters such as dynamic compliance and total lung resistance, and total and differential cell counts, as well as pilot experiments involving analyzing categories of cough “sounds” were measured. The thesis was also concerned with the measurement and classification of cough events and in particular the discrimination of cough events from sneeze events. Two commercially available systems and ad hoc approaches were used to evaluate how best to describe, count and classify the cough response and qualitative and quantitative judgement have been made to assess a best approach.

In summary, the data in this thesis suggests that ozone is a particularly

effective acutely-acting non-allergic sensitising agent capable of shifting the dose response curve of the cough response to citric acid leftward by 0.5 to 1 log units. Sensitization of the cough reflex overcame desensitization of rabbits and guinea pigs to citric acid, allowing cross-over designs to be employed. Ozone appears to act via sensitization of the peripheral airway sensory input, but I found no evidence that this was via an action on Transient Receptor Potential Ankyrin 1 (TRPA1), which has previously been suggested to be an important target for ozone. Codeine and levodropropizine were effective against hypertussive responses, but did not block the normotussive cough. Anticholinergic drugs were not effective against ozone sensitised cough nor normotussive cough responses in the rabbit, but significantly inhibited sensitised cough responses and normotussive cough responses in guinea pigs. However, salbutamol demonstrated a similar treatment profile to the anticholinergic drugs implying that bronchodilation is an important mechanism to reduce the cough response in guinea pigs. Thus, these data suggest that drug candidates that cause bronchodilation may falsely identify as antitussives in the guinea pig model. Phosphodiesterase inhibitors were effective at blocking the infiltration of leukocytes in both guinea pigs and rabbits, but did not effect the acutely sensitised cough, suggesting that in this model ozone is inducing hypertussive responses independently of leukocyte infiltration.

Acronyms

15-HPETE 15-hydroperoxyeicosatetraenoic acid.

5-HT 5-Hydroxytryptamine.

ACCP American College of Chest Physicians.

ACE Angiotensin Converting Enzyme.

ASIC Acid Sensitive Ion Channels.

BAL Bronchoalveolar Lavage.

BHR Bronchial Hyperresponsiveness.

C_{dyn} Dynamic lung compliance.

cAMP cyclic Adenosine Mono-Phosphate.

CAT Catalase.

CI Confidence Interval.

CNS Central Nervous System.

COPD Chronic Obstructive Pulmonary Disease.

COX Cyclo-Oxygenase.

EMG Electromyography.

FFT Fast Fourier Transform.

GABA-B Gamma-Aminobutyric acid subtype B.

GERD Gastroesophageal Reflux Disease.

GIRK G-protein-coupled inwardly rectifying K⁺.

HMOX-1 Heme Oxygenase 1.

i.p. intraperitoneal.

IL Interleukin.

KC Keratinocyte-derived Chemokine.

LED Least Effective Dose.

MCP-1 Monocyte Chemotactic Protein-1.

MIP Macrophage Inflammatory Protein.

MMP-9 Matrix Metalloproteinase Type 9.

MNSOD Manganese Superoxide Dismutase.

NK₁ Neurokinin Receptor Type 1.

NK₂ Neurokinin Receptor Type 2.

NK₃ Neurokinin Receptor Type 3.

NMDA N-methyl-D-aspartate.

NO_x Nitrogen Oxides.

nTS nucleus Tractus Solitarii.

OTC Over The Counter.

PAF Platelet Activating Factor.

PC Percent Change.

PC₃₅ Concentration of agonist in mg/ml required to reduce the dynamic compliance 35% from the baseline.

PC₅₀ Concentration of agonist in mg/ml required to reduce the dynamic compliance 50% from the baseline.

PDE Phosphodiesterase.

PGE₂ Prostaglandin E₂.

R_L Total lung resistance.

RAR Rapidly Adapting Receptors.

s.c. subcutaneous.

SAR Slowly adapting stretch receptors.

shRNA short-hairpin RNA.

TNF Tumour Necrosis Factor.

TPP Trans Pulmonary Pressure.

TRPA1 Transient Receptor Potential Ankyrin 1.

TRPV1 Transient Receptor Potential Vanilloid Type 1.

URTI Upper Respiratory Tract Infection.

VOC Volatile Organic Compounds.

VOCC Voltage Operated Calcium Channels.

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Chapter 1

Innervation of the airways and a review of animal models of cough

Cough is a vital reflex that expels innocuous and harmful particulates and mucus from the airways, protecting the lungs from infection and maintaining patency. However, when cough is persistent, beyond the insult that triggered the cough reflex either when it is greater in frequency or when it is painful then the cough is inappropriate. This inappropriate cough is a disease-state of “hypertussive” coughing resulting from a sensitization to stimuli that triggers the cough reflex; irritants that trigger receptors in the larynx, trachea or the bronchial tree. These regions that can be sensitised can be broadly categorised as either “peripheral” such as sensory nerves in the larynx, trachea or the bronchial tree or “central” such as the nodose and jugular ganglia and proposed ‘cough center’ in the brain (Canning and Spina, 2009).

Hypertussive cough is widely considered to be a grossly unmet clinical need,

it is one of the most common symptoms reported to physicians and severely affects patient quality of life (Dicpinigaitis, 2010). Hypertussive cough can often wake people out of their sleep and the European Community Respiratory Health Survey found that 31% of 18,277 respondents from 16 countries in a Europe wide survey reported they had been “woken by an attack of coughing at any time in the last 12 months” and, of those, almost half reported exclusively suffering nocturnal coughing (Janson *et al.*, 2001). Of those 18,277 respondents 10.2% reported having a non-productive cough and 10% reported having a productive cough (Janson *et al.*, 2001). Further, the European Community Respiratory Health Survey only looked at subjects aged 20 - 48 years old so it is likely that cough prevalence is indeed higher; many chronic cough patients are between the ages of 45 - 58 years old (Ford *et al.*, 2006). The prevalence of hypertussive cough is reflected by patient demand with the market for over the counter cough syrups at £92.5 million in the UK and \$328 million in the US (Morice, 2002; Dicpinigaitis *et al.*, 2014). Chronic cough is considerably more debilitating as it is, by definition, more protracted. The aetiology of chronic cough isn't well understood, but it tends to coexist with asthma, Gastroesophageal Reflux Disease (GERD), Chronic Obstructive Pulmonary Disease (COPD) and upper airway disorders (Birring, 2011). Global prevalence of chronic cough is estimated at 9.4% of the population with a larger proportion of sufferers in Europe, America and Oceania (Woo-Jung Song *et al.*, 2014) and it is associated with numerous diseases that cause the majority of disability-adjusted life years (WHO, 2008). Chronic cough is simply more complex than acute cough, the management and treatment involves a broader

selection of drugs and strategies (Birring, 2011) so this thesis specifically focuses on acute cough because while acute and chronic cough share similarities they are very different conditions.

The standard treatment practice is to treat the underlying cause of the cough and cough suppressants are only used when no such treatments are available or when these treatments are ineffective (Irwin and Madison, 2010). Treating the underlying cause does not always categorically treat the cough symptoms in a significant number of patients (Everett *et al.*, 2007) and, in addition, idiopathic cough may be caused by a variety of unknown factors (Birring *et al.*, 2004). In the case of acute cough, cough is often secondary to a viral Upper Respiratory Tract Infection (URTI) (Irwin and Madison, 2010) and while anti-virals for the common viruses associated with colds, such as acute coryza, have received much preclinical attention (Lewis *et al.*, 1998; Gwaltney *et al.*, 2002; Heikkinen and Järvinen, 2003) there is no effective treatment specifically for the infective agent and thus physicians can only provide symptomatic relief (Heikkinen and Järvinen, 2003; Irwin and Madison, 2010). However, drugs to provide symptomatic relief are far from ideal and indeed the NHS and NIH publicly state that there is no treatment for acute cough (Choices, 2013; NIH, 2014).

The opiates are the primary drug class used to suppress cough with codeine commonly in use in a number of countries (Young and Smith, 2011). However, these drugs have a broad systemic side-effect profile, are physically addictive (Mj, 1996) and their appropriate use has been called into question (Irwin *et al.*, 2006). Dextromethorphan hydrobromide was originally hailed as a non-addictive alternative to codeine (Cass *et al.*, 1954) and has been in wide use

since its discovery as an Over The Counter (OTC) cough suppressant (Tortella *et al.*, 1989). Dextromethorphan binds to two sites in the brain, a low affinity and a high affinity one, and importantly these regions are distinct from opioid and other neurotransmitter sites (Grattan *et al.*, 1995). However, dextromethorphan has demonstrated considerable promiscuity and has been shown to bind to N-methyl-D-aspartate (NMDA) receptors (Ferkany *et al.*, 1988), σ -1 receptors (Meoni *et al.*, 1997; Chou *et al.*, 1999), serotonergic receptors (Meoni *et al.*, 1997) and nicotinic receptors (Glick *et al.*, 2001). It is this broad receptor affinity profile that is thought to explain the mechanism of action for dextromethorphan and one view is that it's primarily acting by altering the threshold for cough initiation via NMDA antagonising glutamate receptors in the nucleus Tractus Solitarii (nTS) (Ohi *et al.*, 2011). Another view is that because σ -1 receptor density is high in the nTS that this may be an important endogenous target (Young and Smith, 2011). Despite widespread use, being actively prescribed for more than 35 years and early clinical trials indicating a significant effect of a 30mg dose of dextromethorphan (Packman and Ciccone, 1983), dextromethorphan use has been called into question. More recent studies that employed objective measures of cough to complement subjective measures failed to find significant efficacy (Lee *et al.*, 2000). In addition, a meta-analysis of dextromethorphan clinical research demonstrated modest 12 - 15% reduction in cough frequency (Pavesi *et al.*, 2001). Correspondingly, dextromethorphan has been put under stricter control by the WHO having been removed from the essential drugs list in 2003; there was insufficient evidence to support dextromethorphan as an essential drug (WHO,

2012). This followed various calls for stricter control from institutions such as the American College of Chest Physicians (ACCP) (Irwin and Madison, 2010).

The ideal antitussive would be anti-*hypertussive*, a drug that could lessen the frequency and magnitude of the cough symptom without abolishing the “hard-wired” cough reflex. Cough hypersensitivity is a key component in the various hypotheses (Millqvist *et al.*, 1998; Fujimura *et al.*, 2000; Prudon *et al.*, 2005; Morice, 2010) for cough aetiology and so it is fitting that the antitussive therapies should be tested in a hypertussive animal model. However, the “gold standard” pre-clinical model to study cough is the guinea pig, either healthy or sensitised by exposure to an inflammatory agent (Brown *et al.*, 2007) or an allergic insult (McLeod *et al.*, 2006). Unfortunately, these models have demonstrated a susceptibility to high “false positive” discovery rates (particularly the use of citric acid challenge in healthy guinea pigs, see 1.6.6) and thus developing an alternative model to the current guinea pig models may lead to a better predictor of antitussive action in man.

1.1 Peripheral sensory innervation of the airways and cough motor responses

The lung contains a range of sensory nerves, as well as receiving innervation from the parasympathetic and sympathetic nervous system (Canning and Spina, 2009) and the activation of sensory nerves primarily elicits the cough reflex (Chung and Widdicombe, 2008).

Table 1.1 summarises the sensory afferents leading from the airways into a

comparative table which has been adapted from (Canning, 2009) and updated with results from primary research papers. The reference to those papers can be found in the reference column of Table 1.1.

The sensory afferents are carried via the vagus nerve and are projected from cell bodies either in the nodose or jugular ganglia. The ganglia process and relay the afferent signals to the Central Nervous System (CNS) and it is here that sensory afferents are thought to summate in a hypothesised “cough centre” in the brain (Canning *et al.*, 2006). The sensory afferent pathways involve two main sensory fibres, C-fibres and A δ -fibres arising from the nodose (mainly A δ -fibres, some C-fibres) and jugular ganglia (both C-fibres and A δ -fibres) and can terminate either at intrapulmonary or extrapulmonary (larynx, trachea and large bronchi) sites (Ricchio *et al.*, 1996).

The various airway afferents and how they summate in the CNS are illustrated in Figure 1.1 and the figure highlights how many redundant pathways are available to elicit a cough response; the different types of sensory nerves and the different types of receptors that can activate those nerves. It is quite possible that the current dearth of antitussive agents is a reflection of the fact that cough has many different excitatory pathways where no single pathway is common to all cough reflexes *or* those pathways that are common to all cough reflexes are difficult targets (e.g. nTS, nodose and jugular ganglia) which by their nature as central targets are likely to be prone to unwanted side-effects. Figure 1.1 and Table 1.1 illustrate the neurosensory anatomy and functional parameters of the peripheral sensory nerves, but the complex interaction between these sensory nerve types and the receptors that mediate their sensory nerve activity, and how

Table 1.1: Characteristics of vagal afferent nerve subtypes innervating the larynx, trachea, bronchi and lungs, adapted from (Canning, 2009)

Anatomical properties	C-fibres							References
	SARs	RARs	Cough Receptors	Neural Crest	Placodal			
Ganglionic origin	Nodose	Nodose	Nodose	Jugular	Nodose			
Intrapulmonary terminations	Yes	Yes	Few	Yes	Yes			
Extrapulmonary terminations	Yes	Yes	Yes	Yes	Few			
Neuropeptide synthesis	No	No	No	Yes	Some			
Physiological Properties								
Conduction Velocity (m/s)	14-32	14-23	4-6	-1	-1			Riccio <i>et al.</i> (1996)
Activity during tidal breathing (impulse/s)	10-50	0-20	N/A	<2	<2			
Lung inflation/stretch	Activated	Activated	N/A	No effect	Activated			
Adaptation to lung inflation	Slow	Rapid	No Effect	No response	Slow			
Lung deflation	No effect	Activated	No response	No effect	No effect			
Carbon Dioxide	Inhibited	No effect	N/A	N/A	Activated			
Acid	N/A	N/A	Activated	Activated	N/A			
Hypertonic Saline	N/A	Activated	Activated	Activated	N/A			
Pulmonary embolism	Sensitized	Activated	N/A	N/A	Activated			
Pulmonary oedema/congestion	Variable	Activated	N/A	N/A	Activated			
Bronchospasm	Activated	Activated	No effect	No effect	No effect			
Pharmacological Properties								
Bradykinin	No effect	Activated	No effect	Activated	Activated			
ATP	Activated	Activated	No effect	No effect	Activated			Undem (2004); Canning <i>et al.</i> (2004)
5-HT	No effect	Activated	No effect	No effect	Activated			
Capsaicin	No effect	Activated	No effect	Activated	Activated			

this relates to transducing the cough reflex, is a topic of ongoing research and debate.

Experimentally, sensory afferent pathways have been classified *in vitro* using parameters listed in Table 1.1 with conduction velocity and impulse activity used as the primary classifying parameters. I have stayed with this tradition of categorising nerves by conduction velocity and impulse activity, but this system of classification, while popular, is not as obvious when trying to classify sensory nerves *in vivo* (Adcock *et al.*, 2014). This broadly limits the translation of *in vitro* single nerve preparations to *in vivo* airway sensory systems. The relative importance of C-fibres and A δ -fibres on the cough reflex have not been elucidated and it is quite likely that their actions are complementary and, in some cases, redundant to one another. This is illustrated by the wide variety of stimuli, the overlap between the ligands that the nerves are sensitive to and the presence of certain receptor types on both of the C-fibres and A δ -fibres and is discussed in the succeeding sections.

1.1.1 Sensory Afferents

C-fibres C-fibres are unmyelinated nerves that respond to both mechanical and chemical stimuli with the exception that the threshold response to mechanical stimuli is higher relative to Rapidly Adapting Receptors (RAR) and Slowly adapting stretch receptors (SAR) (Reynolds *et al.*, 2004). They are defined physiologically by their conduction velocity of 1ms or less (Canning and Spina, 2009) and they terminate at intrapulmonary and extrapulmonary sites (Mazzone *et al.*, 2005).

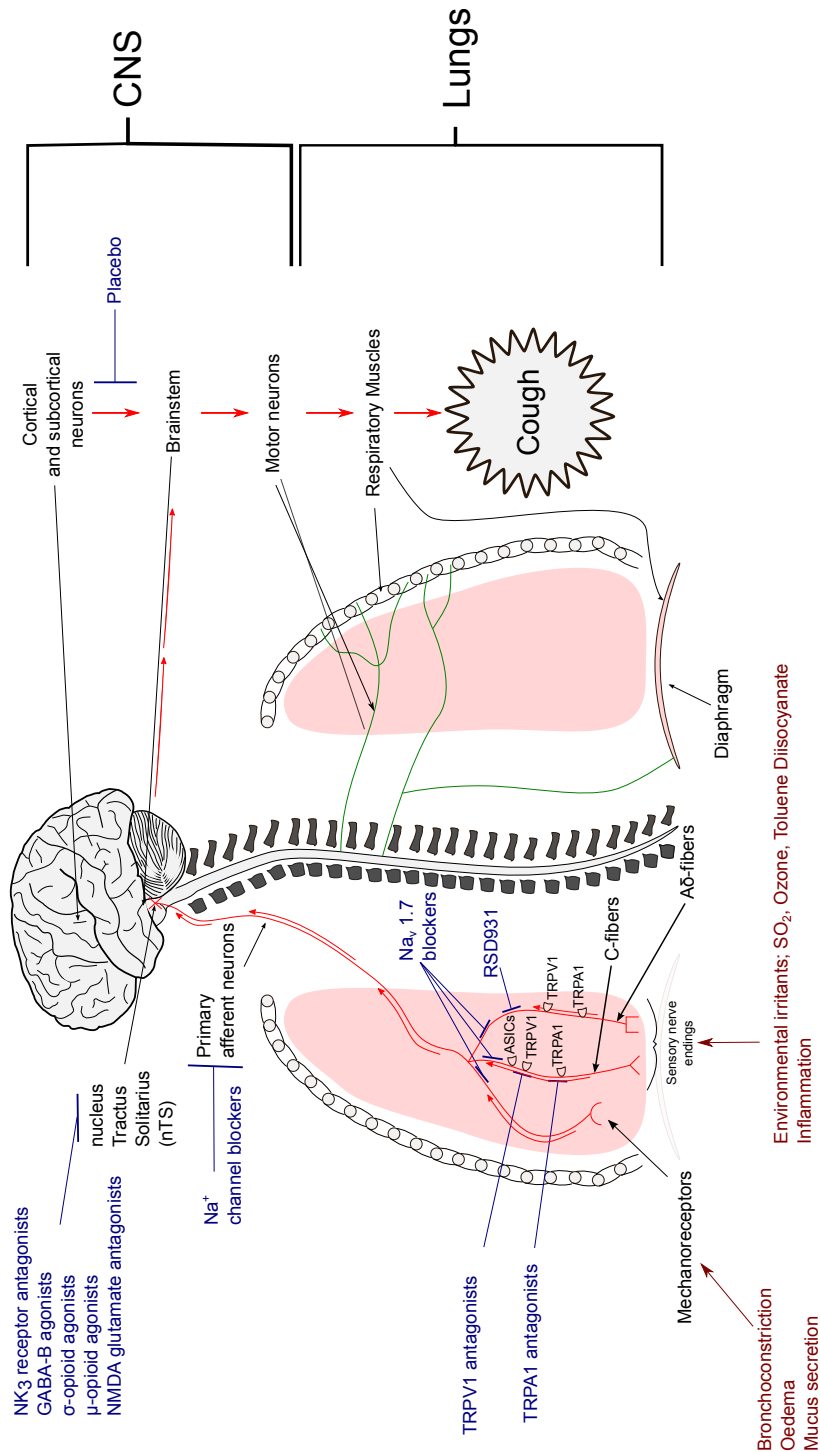


Figure 1.1: Schematic illustration of pharmacological targets for the treatment of cough. The airway afferents are coloured red, the efferent pathways green, the pharmacological targets blue and the endogenous stimuli red. Acronyms are as follows; NK₃ is Neurokinin Receptor Type 3, GABA-B is Gamma-Aminobutyric acid subtype B, TRPA1 is Transient Receptor Potential Ankyrin 1, TRPV1 is Transient Receptor Potential Vanilloid Type 1, SO₂ is sulphur dioxide, ASIC is Acid Sensitive Ion Channels and NMDA is N-methyl-D-aspartate.

C-fibres are particularly important to the cough reflex. They respond to a plethora of chemotussive stimuli in the guinea such as citric acid (Tanaka and Maruyama, 2005), capsaicin (Karlsson, 1996; Leung *et al.*, 2007) and bradykinin (Fox *et al.*, 1996). In addition, C-fibres express a number of receptors that can be involved in the cough reflex, namely, Transient Receptor Potential Vanilloid Type 1 (TRPV1) (Canning *et al.*, 2006), NGF (El-Hashim and Jaffal, 2009) and TRPA1 (Birrell *et al.*, 2009). There is a suggestion that C-fibres may alter the “gain” of the cough reflex and that activation of C-fibres may increase the sensitivity of the airways to coughing (Canning *et al.*, 2004). The response of C-fibres to tussive stimuli is also common between most, if not all, of the animal models of cough as well as humans (Karlsson *et al.*, 1999) illustrating how conserved this mechanism of activating cough is amongst mammalian phylogeny.

C-fibres are tractable, but complex pharmacological targets. Firstly, C-fibres play functionally opposite and complex roles; activation of bronchial C-fibres by citric acid induces a cough reflex (Tanaka and Maruyama, 2005), but when bronchial C-fibres were stimulated by nedocromil in dogs the cough response was suppressed (Jackson *et al.*, 1989). The functional role also differs between species; activation of pulmonary C fibres in the cat inhibits mechanical stimulation of the cough reflex in the larynx (Tatar *et al.*, 1988) and intravenous 5-Hydroxytryptamine (5-HT), known to stimulate pulmonary C fibres in guinea pigs (Hay *et al.*, 2002), inhibits citric acid induced cough in humans (Stone *et al.*, 1993). This effect is possibly due to the fact that C-fibres can arise from nodose or jugular ganglia (Undem, 2004), or it could be that the bronchial C-fibres play a different physiological role than pulmonary C-fibres (Coleridge and Coleridge,

1984; Widdicombe, 1995; Lee and Pissarri, 2001). Secondly, some studies have demonstrated that C-fibres stimulated by capsaicin via activation of TRPV1 receptors do not elicit cough in anaesthetised guinea pigs, while other C-fibre dependent and C-fibre independent mechanisms, such as mechanical and acid stimulation, can (Canning and Spina, 2009). This implies that the cough reflex mediated by C-fibres is complex. It may be the case that if C-fibres could be targeted at a particular branch in the airways (the larynx, bronchi or alveoli), then it may be therapeutically useful, but this represents a difficult drug delivery challenge.

Regardless, levodropropizine and AF-219 have illustrated that C-fibres are important targets for antitussive therapy. Levodropropizine can act on C-fibres in the anaesthetised cat (Shams *et al.*, 1996) and, critically, levodropropizine is clinically efficacious in humans (Catena and Daffonchio, 1997; De Blasio *et al.*, 2012). Similarly, AF-219, a P₂X₃ receptor antagonist, was recently shown to mediate the activity of C-fibres in guinea pigs (Bonvini *et al.*, 2014) and a phase II clinical trial of AF-219 demonstrated clinical efficacy in humans (Abdulqawi *et al.*, 2014).

A δ -fibres, RARs and the Cough Receptor A δ -fibres can terminate either at intrapulmonary or extrapulmonary sites, are mechanically sensitive and, to a lesser degree, chemically sensitive (Mazzone *et al.*, 2005). They are chemically sensitive to changes in osmolality since they respond to distilled water (hypotonic), hypertonic saline and low chloride solutions (Fox, 1995), as well as capsaicin dosages beyond 3 μ M (Fox *et al.*, 1993), citric acid (Canning *et al.*,

2004), histamine (Undem and Carr, 2001) and neuropeptides (Belvisi, 2003). They are chemically *insensitive* to bradykinin and 5-HT (Fox *et al.*, 1993), but as Mazzone *et al.* (2005) points out, A δ -fibres may be indirectly sensitive because these agents can cause mechanical changes via oedema.

A δ -fibres may be important in mediating the cough response and stimulation of RARs is thought to be the most likely cause of cough excitation in the tracheobronchial tree (Widdicombe, 1996a). Inhibition of A δ -fibres by carcanium chloride lead to a decrease in cough response in both guinea pigs and rabbits (Adcock *et al.*, 2003) and this is especially interesting because rarely is the antitussive effect of an agent mirrored so similarly in two species.

RARs are myelinated A δ -fibres throughout the intrapulmonary airways that terminate in close proximity to the epithelium (Widdicombe, 2001). They have conduction velocities between 14-23ms (Ho *et al.*, 2001) and their activity rapidly adapts to stimulation, more rapidly than SARs (Canning *et al.*, 2006). In guinea pigs they respond to a number of chemical stimuli, including bradykinin (Undem, 2004), ATP (Undem, 2004), capsaicin (Adcock *et al.*, 2014) and 5-HT (Undem, 2004). RARs also respond indirectly to capsaicin by acting on C-fibres and stimulating the release of substance P leading to oedema that subsequently stimulates A δ -fibres by mechanical stress. Lastly, these fibres also respond to a variety of mechanical stimuli such as lung inflation (Ho *et al.*, 2001), lung emboli (Armstrong *et al.*, 1976), punctate stimuli (Armstrong *et al.*, 1976) and negative luminal pressure (Bergren, 1997). RARs were originally proposed to be necessary to stimulate the cough reflex (Widdicombe, 1954). However, the cough response is not reduced when human subjects are either treated with

bronchodilators or consciously changing their luminal pressure by forced breathing against a closed glottis (Canning and Spina, 2009). Therefore, RARs are not necessary to stimulate a cough response, but are merely one mechanism that can stimulate a cough response.

A distinct “cough receptor”, a type of $A\delta$ -fibre, was putatively suggested when Widdicombe (1954) first identified the airway afferents in cats (Widdicombe, 1954) and in later studies reported to be a $Na^+/K^+/ATPase$ (Mazzone *et al.*, 2009). The identity of a distinct cough receptor was revisited in work undertaken by Canning *et al.* (2004) in the guinea pig. This putative receptor mediates an axon reflex that conducts slower than $A\delta$ -fibres, but faster than C-fibres, at 4-8 ms and is insensitive to many stimuli apart from acids (Canning *et al.*, 2004) and histamine (Widdicombe, 1954). However, to date I have found no conclusive evidence in the literature of such a cough receptor structure in human lungs similar to what is described in the guinea pig.

SARs SARs similar to RARs are myelinated but conduct in the $A\beta$ -range. They are easily identifiable from their regular discharge in synchrony with transient changes in lung volume and gross movements of inspiration and expiration (Sant’Ambrogio, 1982). They are relatively insensitive to mechanical and chemical stimuli (Widdicombe, 2001) and thus they are of less interest to cough research, but may be applicable to studies where mechanical stimulation of the airway is used to elicit cough.

1.1.2 Receptors on sensory nerves

The sensory afferents express a number of different receptor proteins which when activated can have an effect on the cough reflex either by initiating the cough reflex or sensitising the cough reflex. Some of the receptors identified to date are discussed below:

Transient Receptor Potential Vanilloid Type 1 (TRPV1) TRPV1 receptors are responsive to acid (Canning *et al.*, 2006), lipids such as 15-hydroperoxyeicosatetraenoic acid (15-HPETE) (Hwang *et al.*, 2000) and capsaicin (Trevisani *et al.*, 2004; Flockerzi and Nilius, 2007) and there has been a suggestion that TRPV1 is the cough receptor in humans (Morice and Geppetti, 2004). TRPV1 is a tractable peripheral pharmacological target and TRPV1 antagonists such as carboxamide (McLeod *et al.*, 2006), iodo-resiniferatoxin (Trevisani *et al.*, 2004) and capsazepine (McLeod *et al.*, 2006) antagonise capsaicin and citric acid induced cough in guinea pigs *in vivo*. It is important to note that regular exposure to capsaicin can desensitise airway sensory nerves by depleting substance P containing nerves (Lundberg and Saria, 1983) and this is mediated by activation of TRPV1 (Geppetti *et al.*, 2006; Gazzieri *et al.*, 2007).

However, capsazepine doesn't antagonise cough induced by hypertonic saline implying as, Reynolds *et al.* (2004) points out, that TRPV1 may not be important in defensive cough reflexes, but instead may be useful in mediating hypertussive cough that is the result of TRPV1 receptor sensitization/activation. Furthermore, TRPV1 antagonists have demonstrated a number of side-effects (Wong and Gavva, 2009) with the most serious side-effect reported being

hyperthermia and loss of temperature sensitivity (Gavva *et al.*, 2007). This is likely to restrict their use to serious intractable cough where the patients temperature can be closely monitored. Most recently, a clinical trial of SB-705498, a selective TRPV1 antagonist, failed to significantly reduce cough severity, urge to cough, and cough-specific quality of life scores (Khalid *et al.*, 2014). It is unclear, however, whether the lack of efficacy was due to SB-705498 as a drug or whether TRPV1 antagonism is antitussive in man. It is also possible that SB-705498 would be more effective in patient populations where TRPV1 over-expression is linked to cough hypersensitivity such as cases involving peri and post-menopausal females (Patberg, 2011).

Transient Receptor Potential Ankyrin Type 1 (TRPA1) TRPA1 is an irritant-sensing ion channel expressed in the airway and activated by stimuli such as cigarette smoke, chlorine, aldehydes (Trevisani *et al.*, 2007; Andersson *et al.*, 2008; Canning and Spina, 2009; Lee *et al.*, 2010), reactive oxygen species (Andersson *et al.*, 2008) and lipid peroxidation products (Caceres *et al.*, 2009). TRPA1 responds to this wide range of stimuli by covalent modifications of the cysteine and lysine residues of the receptor on its cytosolic N-terminus, which is somewhat different than the classic key-lock spatial confirmation between agonists Experimentally it has been demonstrated that transfection of an Na_v 1.7 short-hairpin RNA (shRNA) by an adeno-associated virus vector delivered by an injection into the nodose ganglia can greatly reduce citric acid responses in the guinea pig, from a mean of 11 ± cough to 1 ± 2 (Muroi *et al.*, 2011). Pharmacologically, lidocaine is the prototypical sodium channel blocker,

predominately used as a local anaesthetic and effective at reducing cough symptoms clinically (Poulton and Francis, 1979). Currently, lidocaine is being investigated for long-term safety (Lim *et al.*, 2013) and an analogue of lidocaine, GSK-2339345, has been developed as an inhaled voltage-gated sodium channel blocker with picomolar affinity (Kwong *et al.*, 2013). GSK-2339345 has demonstrated an acceptable safety profile in phase 1 clinical studies when compared to placebo and lidocaine (Joanna Marks-Konczalik *et al.*, 2014). A phase 2 clinical study has been planned and is currently recruiting patients (GSK, 2014).

Nicotinic acetyl choline receptors (nAChR) nAChR are activated by cigarette smoke, causing depolarisation of C-fibres and commonly provoke coughing in healthy non-smokers on a single puff of a cigarette (Lee *et al.*, 2010). Lobeline, a nicotinic receptor stimulant has been used in past as a treatment for whooping cough, croup and other respiratory conditions (Millsbaugh, 1892), as well as being used as a smoking cessation agent (Dwoskin and Crooks, 2002), but to date there is very little evidence of researchers considering or using nAChR as a target for an antitussive.

Moreover, nAChR does not represent an ideal target for cough because of the systemic role that nAChR plays in the sympathetic and parasympathetic nervous system. Side-effects such as dizziness, nausea, hypertension, vomiting, stupor, tremors, paralysis, convulsions and coma are all related to the action on the sympathetic and parasympathetic nervous system; this greatly limits the effectiveness of nAChR antagonists.

E Series Prostanoid receptor 3 (EP₃) EP₃ receptors are activated by Prostaglandin E₂ (PGE₂) and transduced by the vagus nerve (Maher *et al.*, 2009). They act directly to cause cough and EP₃ deficient mice lack any vagal activity when exposed to PGE₂ (Maher *et al.*, 2009). Furthermore, humans challenged with PGE₂ (0.1 - 100 µg ml⁻¹) cough between 4 to 10 times within 1 minute of the aerosol challenge (Costello *et al.*, 1985). There is also evidence that suggests that PGE₂ can sensitise TRPV1 (Kwong and Lee, 2002) pathways indicating that PGE₂ may play a role in both triggering a cough and sensitising the cough reflex.

The tractability of EP₃ as an antitussive target is unclear given that moderate doses of aspirin can suppress Angiotensin Converting Enzyme (ACE) inhibitor-induced cough (Tenenbaum *et al.*, 2000), but selective Cyclo-Oxygenase (COX)2 inhibitors do not have any effect on the cough reflex (Dicpinigaitis, 2001). Nonetheless, COX inhibitors have been in general use long enough that if there were a relationship between COX inhibitor use and cough suppression then it would likely have been observed.

1.2 Central modulation of the cough response and the urge-to-cough

The understanding of the central regulation of cough is improving, but is far from complete. Borison (1948) first reported that stimulating the dorsolateral region of the medulla oblongata can lead to coughing and later, using histological techniques, detected that neural substrates resulting from stimulating the cough reflex electrically, tended to be found in the rostral pons Dubi (1959). Experiments based on stimulating the dorsolateral region of the medulla oblongata followed. Chakravarty *et al.* (1956) used this protocol and administered codeine and dextromethorphan before decerebrating cats and thus concluded that codeine and dextromethorphan were acting on central targets. It was later shown that codeine and dextromethorphan act on both central *and* peripheral targets (Adcock *et al.*, 1988). Chou and Wang (1975) were perhaps the first to attempt to localise the central cough pathways in the vertebrae by electrically stimulating the lower brainstem regions of the cat as well as attempting to refine the “Cough center” region described by Borison (1948). In addition, Chou and Wang (1975) comprehensively tested a number of antitussives including caramiphen ethanedisulfate, codeine, dextromethorphan, clonazepam and benzonatate. Broadly, the dose required was 1/20th of that required intravenously and, specifically, clonazepam was the most efficacious antitussive; roughly 12 times more effective than dextromethorphan (Chou and Wang, 1975). The cough motor pattern is thought to be regulated in a different manner than the breathing motor pattern and

attempts have been made to model this division (Shannon *et al.*, 1998; Bolser and Davenport, 2002). These models have been extended to include a network model for the control of laryngeal motoneurons within this framework (Baekey *et al.*, 2001), although the validity of these models has yet to be demonstrated *in vivo*. Downstream from the cough center, there is considerable interest in targeting ganglia downstream of the cough center, with notable targets being the nTS and the nodose ganglia (Baekey *et al.*, 2003; Ohi *et al.*, 2005; Mutolo *et al.*, 2007). The nTS and the nodose represent the junctional terminus for many of the neuronal projections into the lung and at this site, it is proposed, a state of plasticity can determine the sensitivity of the organism to tussive stimuli (Bonham *et al.*, 2006). Dextromethorphan is thought to act by antagonizing glutamate receptors in the nTS (Ohi *et al.*, 2011), indicating that it is a viable central target for cough therapy.

Aside from the neurophysiology of the cough reflex, there is a great deal of interest in the “urge-to-cough” - the sensation of knowing that you need to cough preceding the actual cough motor response. The urge-to-cough has been studied, with great interest, in smokers and in smoking cessation studies with the administration of nicotine gum greatly reducing the reported intensity of urge-to-cough (Davenport *et al.*, 2009). In a review, Widdicombe *et al.* (2011) illustrated that there are many influences on the urge-to-cough; intranasal and oral administrations, cognitive behaviour techniques and breathing techniques all show efficacy. Oral administrations of honey had a clear antitussive action in children with acute cough (Paul *et al.*, 2007) and has been robustly confirmed in an appropriately powered double-blind, randomized, placebo-controlled study

(Cohen *et al.*, 2012). Cohen *et al.* (2012) arguably answers the criticism that earlier trials were under powered trials (Schroeder and Fahey, 2002). Oduwole *et al.* (2014) in a review of randomised controlled trials concluded that honey was indeed better than no treatment (mean difference (MD) -1.07; 95% confidence interval (CI) % -1.53 to -0.60; two % studies; 154 participants) with evidence suggesting that the antitussive effect of honey did not significantly differ from that of dextromethorphan (MD -0.07; 95% CI -1.07 to 0.94; two studies; 149 participants). There is suggestion that the sweet flavour sensation of honey is the most important aspect of these effects and is a probable reason why most of the OTC medicines are sweetened (Wise *et al.*, 2014). The key issue is that cough is greatly influenced by the placebo effect and as much as 85% of the antitussive effect is attributed to the placebo effect (Eccles, 2006).

Modern functional studies using fMRI has been used to identify which regions of the brain respond to airway irritation and which areas of the brain that are activated before coughing occurs (Mazzone *et al.*, 2007; Mazzone *et al.*, 2011). Primary motor and somatosensory cortices and the posterior mid-cingulate cortex were common regions activated by evoked cough (Mazzone *et al.*, 2011) and this demonstrates that there are neurophysiological events that correspond with the urge-to-cough extending what was first established in original experiments by Chou and Wang (1975). The number of different functional regions activated illustrate that there are many functional processes involved in the cough reflex such as processing the afferent inputs, projecting a perceptual experience and planning and engaging the motor responses. Recent work by Farrell *et al.* (2014) identified multiple “seed” regions,

regions that pre-empt the motor responses and it was concluded that these distributed regions form a subnetwork that control for cough suppression, stimulus intensity coding and the perceptual components of urge-to-cough.

1.3 The definition, mechanism and measurement of cough

The definition and subjective measure of cough

There is no universally agreed definition of what constitutes a cough, but attempts have been made to reach a definition by consensus (Morice *et al.*, 2007). The audible sound produced by the cough is considered the signal modality of primary importance, with secondary modalities such as EMG of the diaphragm (Lunteren *et al.*, 1989; Bolser *et al.*, 1999) and intra-thoracic pressure (Xiang *et al.*, 1998) being used to confirm the sound heard. These attempts, however, have failed to produce a definitive, objective measure of cough because cough is too varied and heterogeneous to satisfy a succinct definition. Rather, the ERS committee came to two clinical definitions of cough as either:

A three-phase expulsive motor act characterised by an inspiratory effort (inspiratory phase) followed by a forced expiratory effort against a closed glottis (compressive phase) and then by opening of the glottis and rapid expiratory airflow (expulsive phase).

or:

A forced expiratory manoeuvre, usually against a closed glottis and associated with a characteristic sound.

These definitions will serve as the basis of my subjective measure of cough throughout this thesis. Further, “cough response” will refer to the number of coughs recorded within the given protocol period.

Objective measures of cough

Different signal modalities assessed in measuring cough Normotussive individuals do not cough regularly and cough as a symptom of disease can be varied, both in frequency and magnitude, and idiosyncratic. It is common, therefore, to provoke cough by means of inhalation of a suitable irritant and measure the number of coughs elicited within an acute period after the provocation such as 10 - 15 minutes, this means of inducing cough was first published by Bickerman and Barach (1954).

Attempts were made by others to record the cough sound or other modalities as early as 1937, when Coryllos (1937) used a manometric recording by means of an inserted catheter to measure the intrapleural pressure during a cough. A similar method used a Grass Transducer to write ink on paper (Gravenstein *et al.*, 1954). Later Gravenstein *et al.* (1954) used an inflated balloon under the mattress of symptomatic patients to transduce a signal, but recording the cough sounds and studying the waveform therefore doesn't appear to have occurred until Woolf and Rosenberg (1964) did so with a magnetic tape recorder.¹

¹The advent of the magnetic tape recorder came in 1928 and became generally available after World War II but I can't find any evidence that it was used to record cough before this point.

Measuring the EMG of the diaphragm as a means of determining a cough event has also been described (Cox *et al.*, 1984) and Figure 1.2 illustrates her experimental setup.

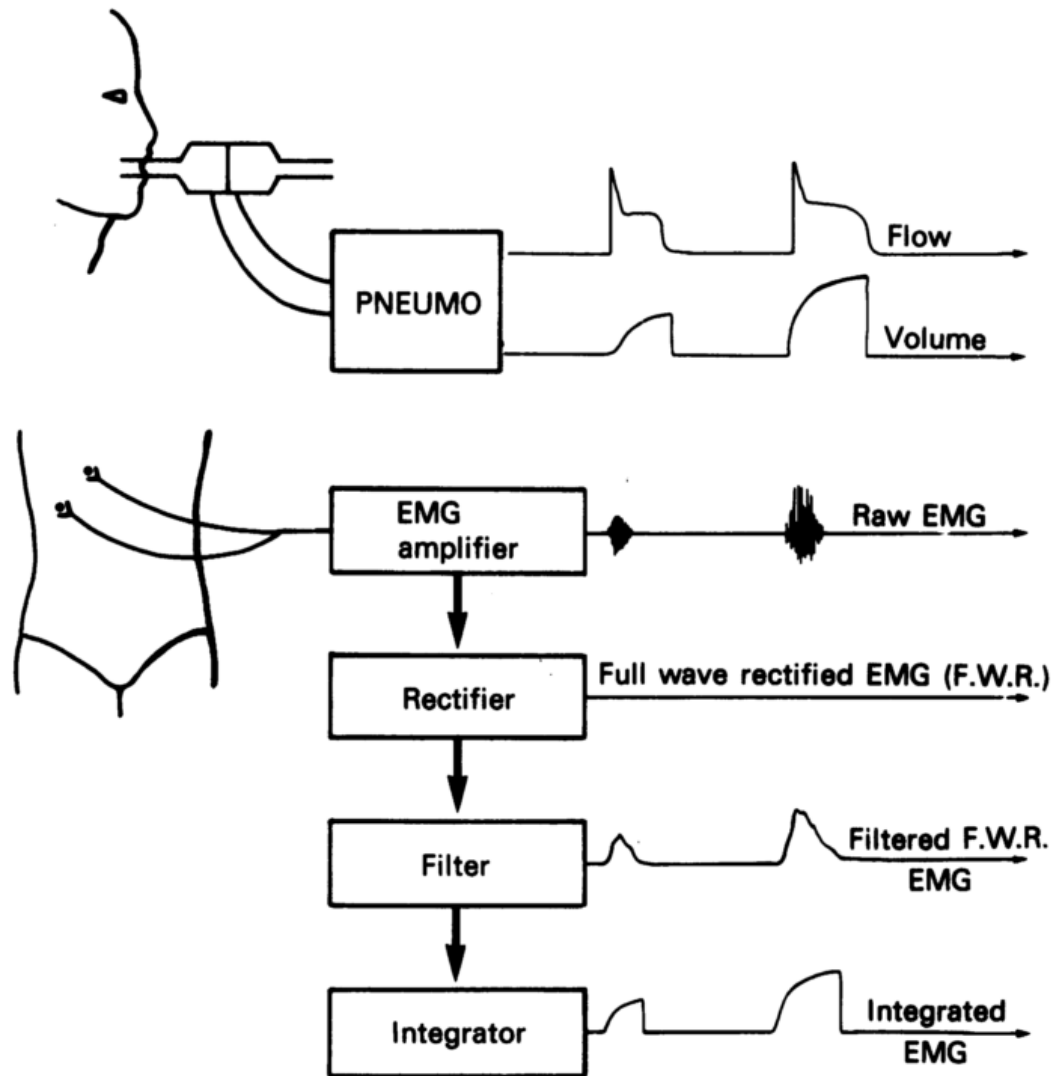


Figure 1.2: Diagram of the method for recording EMG and cough airflow with sample traces, the wave rectified trace is not shown. Reproduction of figure 1 from Cox *et al.* (1984)

Spectral analysis of the cough sound Critical to the understanding of the sound waveform is the basic principle that the information of the sound is *not* in the time domain but rather in the frequency domain. The human ear decodes sounds by using thousands of hair cells in the ear each receptive to a particular frequency. From this orchestra of frequencies the brain computes the meaning of the sound. It is of interest, therefore, to be able to analyse sound as our ear analyses sound and for this we must convert the time domain of the signal into the frequency domain.

Translation of the time domain signal into a frequency domain signal is done by the means of a Fourier Transform, the transform is able to do so based on the assumption that all complex signals can be described by a number of base frequencies scaled by their amplitude (Smith, 2003). The common computer implementation of the Fourier Transform is the Fast Fourier Transform (FFT)(Cooley *et al.*, 1964).

Korpás has made extensive use of FFT to define different components of cough and components of cough that associate with a variety of disease states (Korpás *et al.*, 1996). Korpás' work involves some thousand separate 'tussigrams' of human cough and further to this he has measured many contemporaneous modalities such as the audible sound, the state of the glottis, oesophageal pressure and airway flow. 1.3 illustrates various modalities and how they affect the prototypical cough.

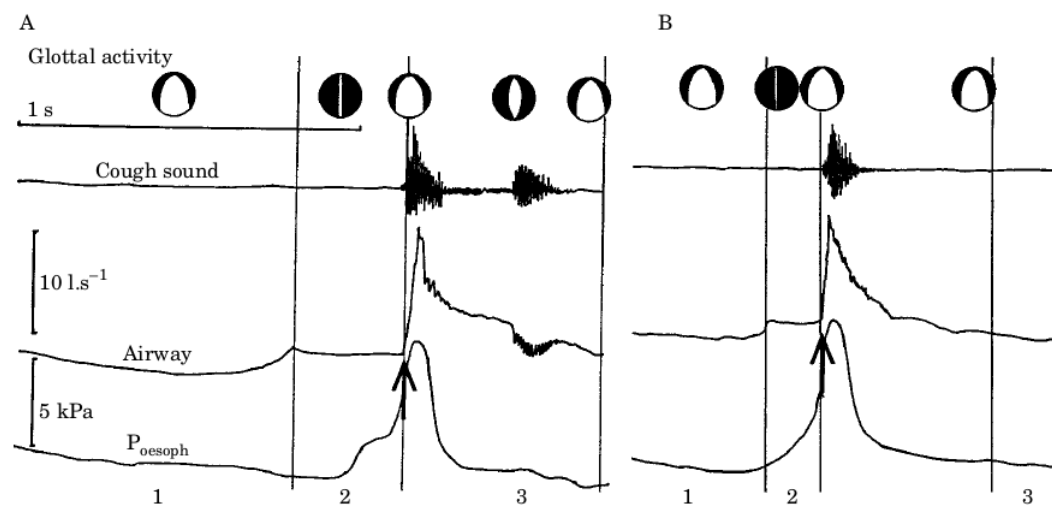


Figure 1.3: The glottal activity, time, records of cough sound, airflow, and oesophageal pressure (inspiration is downward) during a single cough (↑) in a healthy subject. 1: Inspiratory cough phase, 2: Compressive cough phase, 3: Expulsive cough phase. Time bar = 1s. (A) Cough with double sound, (B) Cough with single sound. Adapted from figure 1 of Korpás *et al.* (1996)

Further, on comparison of the sound waveform and these other modalities he categorised particular components to particular disease states, illustrated in figure 1.4. He noted a longer and louder sound for those subjects with mild bronchitis and an even longer and louder cough sound with those with severe bronchitis. Korpás also identified differences in the frequency spectra and was able to demonstrate that patients with chronic bronchitis had their spectra skewed left towards lower frequencies and that normal coughers had a spectra that was skewed towards higher frequencies, see fig. 1.5.

This author considers the analysis of the frequency spectra of great importance in the objective measurement on cough.

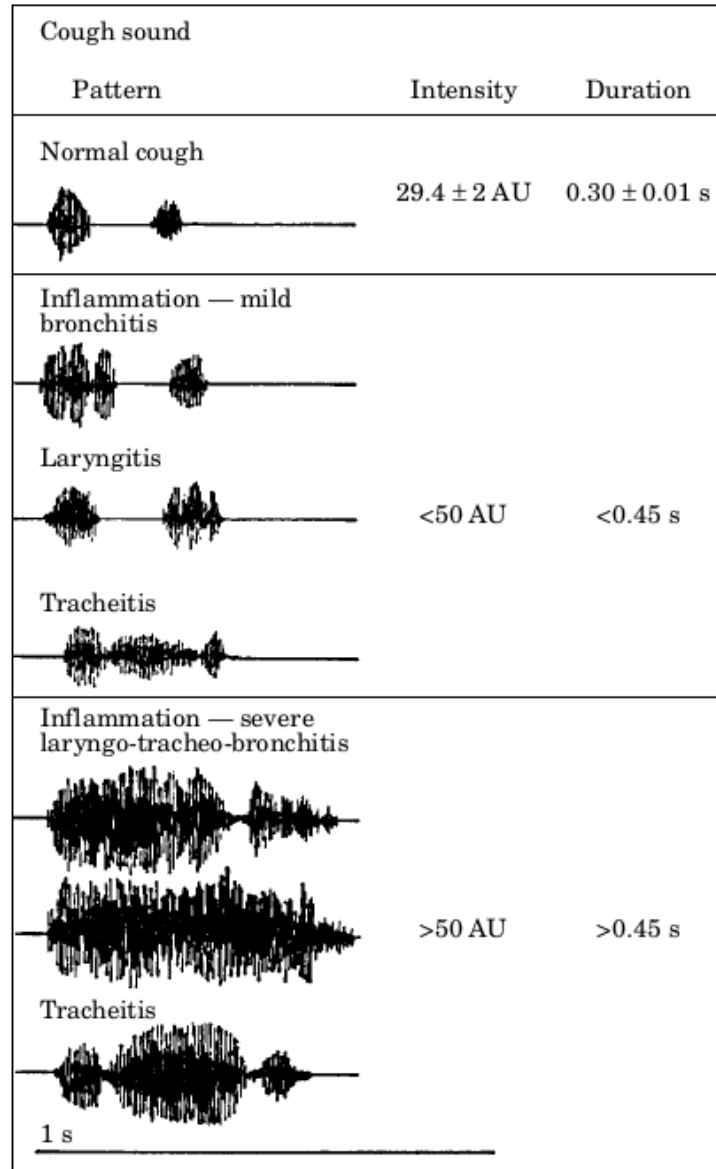


Figure 1.4: Changes of the cough sound pattern, intensity and duration in a healthy subject, a subject with mild inflammation and subject with severe inflammation. The values in the normal cough represent the mean \pm SEM, in mild inflammation they represent the maximal limit of the normal cough and in “severe inflammation” they represented the threshold of the disease values. The time bar is equal to 1s, this figure is a re-production from figure 2 of Korpás *et al.* (1996)

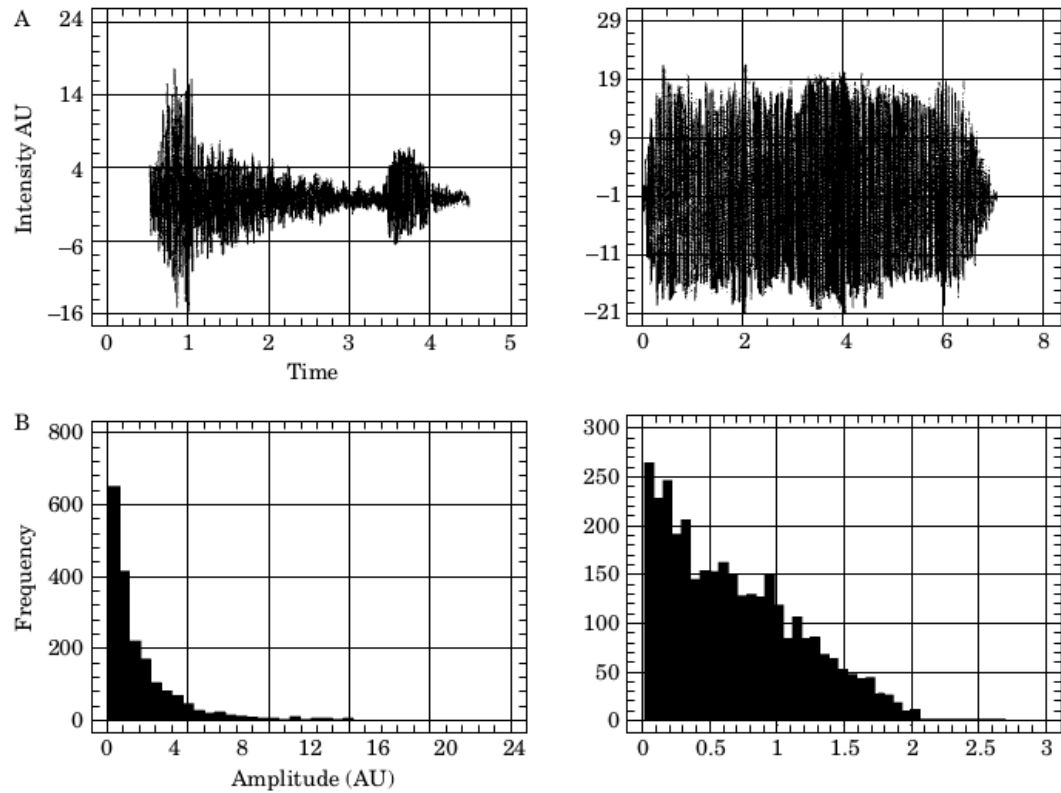


Figure 1.5: The cough sound recorded in healthy subjects (A, left) and a patient with chronic bronchitis (right). A histogram of the samples of sound amplitude (amplitude, arbitrary units, AU) according to their frequency occurrence ((B), frequency). The trend of the bar charts approximates a hyperbola in normal subjects and approximates a linear response in subjects with chronic bronchitis. The cough sound was generated by exposing the subject to a nebulised dose of 10% citric acid. This figure is a reproduction of figure 3 from Korpás *et al.* (1996)

1.4 Sensitization of the cough reflex

The cough reflex can be sensitised by a number of events; inflammation in diseases such as COPD (Smith and Woodcock, 2006), allergy such as in asthma (Chang and Gibson, 2002) and without a specific cause in the case of idiopathic cough (McGarvey and Ing, 2004). The specific mechanism of how cough is sensitised is not well understood and this is a reflection of the fact that cough is the result of complex lung-brain interactions (Smith and Woodcock, 2006), somewhat analogous to how pain is a complex of peripheral-brain interactions (Adcock, 2009).

Inflammatory conditions lead to many pathological changes around and within airway sensory nerve fibres. This leads to increased excitability of airway afferents as well as phenotypic changes in receptor and neurotransmitter expression (Reynolds *et al.*, 2004). Clinically, it has been extensively documented that patients with chronic airway inflammation (typical in diseases such as COPD, asthma, eosinophilic bronchitis, and URTI) have a larger cough response to capsaicin (Chung and Lalloo, 1996; O'Connell *et al.*, 1996; Doherty *et al.*, 2000). Preclinically, there are a number of physiological features that have been correlated to airway inflammation. Mechanosensitive A δ -fibres under physiological conditions do not contain neuropeptides but following viral and/or allergen challenge they start to synthesize neuropeptides (Carr *et al.*, 2002). In addition, the excitability of airway A δ -fibres and nTS neurons can be increased by antigen stimulation (Undem *et al.*, 2002). There is a key notion that it is the “plasticity” of airway neurons mediating the cough response that leads

to the sensitization of the cough reflex. Indeed, persistent inflammatory conditions are considered to be a key component of chronic cough that causes observable structural and pathological changes to the airways (Niimi, 2011). Inflammation can be triggered preclinically by exposing a subject to an appropriate inflammatory agent such as LPS. LPS ($50 \mu\text{g ml}^{-1}$, intratracheal) significantly reduced the time taken to cough to citric acid in guinea pigs (Brown *et al.*, 2007). In addition, dexamethasone prevented LPS induced neutrophilia, but not hyperresponsive cough indicating that sensitization of the cough response was not dependent on neutrophils (Brown *et al.*, 2007). In our study, LPS was chosen as a sensitization agent in the guinea pig model using the same dose as Brown *et al.* (2007).

Allergy is an exaggeration of the immune system to specific antigenic stimuli and involves an adaptive immune response. However, the aetiology has a lot of overlap with the inflammatory sensitization of the cough reflex which is predominately an innate immune response. Jinnai *et al.* (2010) used a series of histological examination of biopsies, autopsies, lung function and CT imaging to assess the pathological changes in the airways in the lungs of healthy, non-asthmatic and asthmatic coughers. One observation was a proliferation of goblet cells and it was proposed that this leads to hypersecretion of mucus and thus a greater cough response to clear the airways (Jinnai *et al.*, 2010). Another observation was that TRPV1 receptors proliferated leading to a greater response to tussive stimuli when patients were exposed to capsaicin (Jinnai *et al.*, 2010). The ovalbumin sensitised guinea pig is a well-established allergic model of cough (Featherstone *et al.*, 1988; Hj *et al.*, 2004; McLeod *et al.*, 2006; Mokry and

Nosalova, 2007). It involves immunizing guinea pigs over a 28 day period with injections of ovalbumin at regular 7 day intervals coupled with concomitant administration of aluminium hydroxide as an adjuvant (McLeod *et al.*, 2006). In this study, we have not investigated an allergic model of cough and this is partly because the protocols are longer and there is high failure rate; as many as a third of guinea pigs sensitised to ovalbumin can die from anaphylaxis (Hoshiko and Morley, 1993). In addition, it would be more beneficial to focus on finding a non-allergic method of sensitising the airways because it could lead to the discovery of a common sensitization pathway to explain cough hyperresponsiveness.

Idiopathic cough is where the cause of the cough remains undetermined despite a systematic evaluation, however, commonly patients are female and peri or post-menopausal (McGarvey and Ing, 2004). McGarvey and Ing (2004) in a 2004 review lists various hypotheses and case studies of idiopathic cough and he proposes that idiopathic cough could be the result of an autoimmune disease with hypothyroidism, coeliac disease, vitiligo and pernicious anaemia all associated with idiopathic cough as well as the aforementioned female gender bias implicating hormonal and age-related effects. There is evidence to suggest that over-expression of TRPV1 is linked to the shift in cough hypersensitivity (Patberg, 2011) and this may be a therapeutically useful target in patients with idiopathic cough. In relevance to this thesis, idiopathic cough is an interesting cause of cough, but not particularly tractable as the basis of developing a preclinical model of cough.

In this thesis, we've chosen to focus on the ozone sensitised rabbit model of

cough, it was first described by Adcock *et al.* (2003). Acute exposure to ozone can greatly sensitise rabbits to citric acid induced cough and further, they can sensitise rabbit from a state of insensitivity to being sensitive to citric acid. The mechanism by which ozone sensitises the airways is not known, but it may have a specific mechanism by acting on TRPA1 to sensitise sensory afferents to tussive stimuli (Taylor-Clark and Udem, 2010). However, it is also well established that ozone acts non-specifically because it is a powerful oxidant and thus will bind to and damage the airway epithelia and this is observable in increased protein expression of Catalase (CAT), Heme Oxygenase 1 (HMOX-1) and Manganese Superoxide Dismutase (MNSOD) (Islam *et al.*, 2008; Gibbs-Flournoy *et al.*, 2013). Critically, the time course of inflammation is delayed and the inflammation occurs *after* Adcock *et al.* describes the sensitization of the airways to cough. Thus, ozone sensitization may represent a non-allergic, non-inflammatory sensitization agent.

1.5 Peripherally acting antitussive pharmacotherapy

A number of drug classes have been investigated as potential peripherally acting antitussives, but there are no recent peripherally acting antitussives that has been approved for clinical use. table 1.2 lists both peripherally and centrally acting antitussives that have recently been studied clinically. A recent review about the state of antitussives has identified a number of new promising drug classes, as well as drugs that are currently used as primary antitussives, such as opiates and local anaesthetics, or secondarily to address cough stimuli such as

mucus hypersecretion, with mucolytics such as guaifenesin (Dicpinigaitis *et al.*, 2014). In my thesis, a major focus was on anticholinergic drugs using tiotropium bromide and two analogues of tiotropium bromide, CHF-5843 and CHF-6023. This was to assess whether anticholinergic drugs could act as peripherally acting acute antitussives. A range of peripherally-acting antitussives were included to act as positive controls (codeine) and negative controls for bronchodilation (salbutamol) and leukocyte recruitment (roflumilast).

1.5.1 Opiates and opioids

Opiates, and the synthetically derived opioids, are one of the oldest drug classes associated with cough (Dicpinigaitis *et al.*, 2014) with reports on the effect of inhaled opiates on “catarrhus” cough published some 200 years ago (Mudge, 1779). Codeine, a methylated derivative of morphine, has been commonly used as an antitussive for around 150 years entering usage when it was isolated from opium by H. Martín in 1834 (Eddy *et al.*, 1969) and has been considered a “gold standard” in antitussive therapy (Doona and Walsh, 1998; Karlsson *et al.*, 1990; Chung, 2003). Codeine acts on both peripheral and central targets. Centrally, Chou and Wang (1975) demonstrated the central effect of codeine by intravertebral injection in anaesthetised cats and stimulating the cough response by electrical stimulation of the region dorsomedial the trigeminal tract. In seven of the eight preparations 0.02 mg kg^{-1} was sufficient to abolish the cough reflex, 1 hour after the injection the cough response was restored (Chou and Wang, 1975). Similar experiments, acting centrally but inferior to the region Chou and Wang (1975) used, injected codeine into the caudal ventral

Table 1.2: List of antitussive drugs and the state of their current clinical development.

Drug	Mechanism of Action	Stage	State	References	Clinical Trial number
Lidocaine	Sodium channel blocker	Phase 4	Ongoing	Lim <i>et al.</i> , 2013	NCT01252225
AF-219	P2X3 antagonist	Phase 2b	Ongoing	ClinicalTrials.gov, 2014a	NCT01432730
VRP700	Unknown but structurally similar to lidocaine	Phase 2b	Ongoing	LSE, 2014	
GSK 2339345	Sodium channel blocker	Phase 2	Ongoing	GSK, 2014	NCT01899768
SB-705498	TRPV1 antagonist	Phase 2	Failed	Khalid <i>et al.</i> , 2014	
Sibenadet	D ₂ -dopamine receptor agonist / β_2 -receptor agonist	Phase 2	Failed	Ind <i>et al.</i> , 2003	
CP-99,994	Neurokinin Receptor Type 2 (NK ₂) receptor antagonist	Phase 1	Failed	Fahy <i>et al.</i> , 1995	
Pirfenidone	Unknown	Phase 1	Ongoing	ClinicalTrials.gov, 2014b	NCT02009293

respiratory column and observed a 29% decrease in cough number and 33% decrease in cough magnitude (Poliacek *et al.*, 2010). Peripherally, Karlsson *et al.* (1990) illustrated that inhaled codeine could also reduce the cough response, in a dose-dependent manner, by 40% at 3 mg ml⁻¹ and 80% at 10 mg ml⁻¹ in guinea pigs. Similar results were demonstrated a 50% reduction in the cough response when codeine, 10 mg kg⁻¹, was administered intraperitoneal (i.p.) or or subcutaneous (s.c.) (Kotzer *et al.*, 2000).

Mechanistically, opioids are well known for their analgesic properties mediated primarily by μ -opioid receptors. Opioids also demonstrate antitussive properties which are also mediated by μ -opioid receptors along with contributions from δ -opioid and κ -opioid receptors (Kamei, 1996). μ -opioid and δ -opioid receptors are G-coupled receptor proteins (G_o/G_i) and activation of these receptors leads to the opening of potassium channels (via a cyclic Adenosine Mono-Phosphate (cAMP) mediated pathway), closing of Voltage Operated Calcium Channels (VOCC) and a reduction in the action of cAMP via stimulation of phosphodiesterases (McDonald and Lambert, 2005). κ -opioid receptors are similar to μ -opioid and δ -opioid receptors, but cause a decrease in potassium conductance (Page *et al.*, 2006) rather than an increase in potassium conductance. The net effect reduces neuronal excitability and is characterised by a reduction of nerve impulses and a reduction in the release of neurotransmitters. μ -opioid receptors can be found on nociceptive sensory nerves, at both pre-synaptic and post-synaptic termini. Endogenous and exogenous (i.e. morphine, codeine) ligands act as analgesics by reducing the transmission of nociceptive stimuli to the central nervous system. μ -opioid

receptor agonists are important in mediating antitussive effects; it has been shown that naloxone, a selective μ -opioid receptor antagonist, can block the antitussive effect of codeine in guinea pigs (Adcock *et al.*, 1988) and rabbits (Simera *et al.*, 2013). Further, BW443C, a selective μ -opioid receptor agonist that doesn't pass the blood-brain-barrier, significantly reduced the cough response without depression of ventilation when administered subcutaneously (Adcock *et al.*, 1988) indicating that opioids can act on peripheral opioid receptors and therefore the choice of opioid can mitigate against central side-effects such as respiratory depression. δ -opioid and κ -opioid receptor agonists can reduce excitatory post-synaptic potential in the nTS in rats, but to a lesser extent than μ -opioid receptor agonists (Rhim *et al.*, 1993). This may contribute to antitussive activity as many of the sensory nerve projection into the airways originate from the nTS (see section 1.1). Kamei *et al.* (1989) has shown that DPDPE, a selective δ -opioid receptor agonist, can mediate the action of μ -opioid receptor and κ -opioid receptors, however, the experiments are conducted in rats and thus the results may be implausible; it is considered that rats either can't cough (Korpás and Kalocsayová, 1975) or that the objective assessment of cough in rats is difficult (Takahama and Shirasaki, 2007). Regardless, single neuron experiments in rats have illustrated that naltrindole and naltriben, two potent δ -opioid receptor antagonists, can activate G-protein-coupled inwardly rectifying K⁺ (GIRK) channels and contribute to a negative feedback loop for 5-HT release. Knowing that pizotifen, a 5-HT₂/5-HT₁ receptor antagonist, can inhibit morphine-induced antitussive effect in humans (O'Connell, 2002) then it is possible that δ -opioid receptor agonists may

enhance opioid-induced antitussive effects by potentiating this serotonergic pathway (Takahama and Shirasaki, 2007).

The routine use of codeine as a prototypical antitussive has been criticized (Schroeder and Fahey, 2002; Reynolds *et al.*, 2004; Bolser and Davenport, 2007; Dicipinigaitis *et al.*, 2014). In a systematic review by Schroeder and Fahey (2002), only two trials out of the five that were assessed demonstrated significant efficacy when compared to placebo. The first of these two studies investigated codeine treatment for coughs that resulted from URTIs and found that oral administration of codeine (30 mg kg^{-1}) for a period of 5 days was no more effective than placebo syrup when measured using objective cough recordings on the first day or self-reported cough scores during the whole 5 day period (Eccles *et al.*, 1992). The second of these two studies was similar to the first, by the same group, and confirmed this lack of efficacy (Freestone and Eccles, 1997). Codeine and opioids, in general, are not ideal antitussives because broad-spectrum opiates such as codeine act on targets both centrally and peripherally and as such have a well known list of undesirable systemic side-effects including sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance, and respiratory depression (Benyamin *et al.*, 2008). This can be offset by choosing an opiate such as hydrocodone bitartrate (dihydrocodeinone), a peripherally-acting selective μ -opioid receptor agonist, over codeine to reduce gastrointestinal and neuro-psychological side-effects (Doona and Walsh, 1998) or consider using opiates that are selective for δ -opioid receptors to reduce constipation and opiate addiction (Kamei, 2002; Chung, 2003).

However, codeine is a useful drug in our study as it has been shown to significantly reduce the cough response in rabbits and guinea pigs (Karlsson *et al.*, 1990; Bolser *et al.*, 1994; Kotzer *et al.*, 2000; Adcock *et al.*, 2003; Simera *et al.*, 2013). It is an appropriate treatment to use as a positive control for antitussive pharmacotherapy.

1.5.2 Anticholinergics

Anticholinergics are a class of drugs that target muscarine receptors and broadly these receptors are associated with signaling the parasympathetic nervous system. In the respiratory system, antimuscarinics are commonly used as bronchodilators by antagonising the activation of muscarinic receptors on airway smooth muscle leading to relaxation of the smooth muscle and mucous glands leading to the reduction of mucous secretions (Page *et al.*, 2006). There are numerous subtypes of the muscarine receptors (M_1 , M_2 , M_3).

It has been suggested that anticholinergics are antitussive, an observation from clinical trials involving COPD patients (Casaburi *et al.*, 2002; Hasani, 2004; Tashkin *et al.*, 2008). The UPLIFT study was a key study supporting the hypothesis that muscarinic antagonists have secondary pharmacodynamics to bronchodilation and amongst various quality of life scores it was observed that there was a reduction in the frequency of cough exacerbation (Bateman *et al.*, 2009). The UPLIFT study was a four-year study with two arms; a tiotropium bromide group and a placebo group. Both groups were permitted to use all respiratory medications except inhaled anticholinergic drugs. At the end of the four year period, patients in the tiotropium group had a demonstrable

reduction in the number of exacerbations, related hospitalisations and respiratory failure as well improvements on the St. George's Respiratory Questionnaire (SGRQ) (Tashkin *et al.*, 2008). However, the results of the UPLIFT study contrasts with a 1 year study in 2002, two years before the UPLIFT study, that specifically identified that while there was an improvement in shortness of breath and wheezing that cough and chest tightness did not improve when compared to placebo (Casaburi *et al.*, 2002). In a smaller study focusing more intensely on cough in COPD patients, Hasani (2004) demonstrated a moderate decrease in cough frequency (27 ± 6 to 19 ± 5 ; mean \pm range) over a 6h period with COPD patients given tiotropium bromide. Dicipinigaitis *et al.* (2008) has also shown that tiotropium bromide can cause a significant decrease in C₅ (the dose of tussive agent required to cause five coughs) to capsaicin-induced cough, however some groups have raised concerns that C₅ does not correlate with objective cough measures (Satia *et al.*, 2014). The evidence that tiotropium bromide is antitussive, therefore, is varied, contrasting and modest at best, perhaps reflecting the variability of the underlying aetiology of COPD, but suggestive that tiotropium bromide could be antitussive.

Bateman *et al.* (2009) proposed a number of mechanisms by which tiotropium bromide could contribute to the reduction to cough exacerbations. Firstly, tiotropium bromide may be antagonising receptors and chemokines that lead to the production of mucus and in doing so reduce cough symptoms because coughing is initiated to expel objects that might obstruct airflow. Secondly, tiotropium bromide may be immunomodulatory and by reducing inflammation and thereby reduce irritation of the airway epithelia this could

reduce the urge to cough.

In humans, tiotropium bromide may assist mucociliary clearance; while tiotropium bromide appears to be ineffective on mucociliary clearance acutely (Hasani, 2004), significant effects on mucociliary clearance can be observed 14 days after treatment (Meyer *et al.*, 2011). Similarly, methoctramine, an M₂-muscarine antagonist, can reduce the production of mucus secreting cells in the airways of ferrets, reducing the production of mucus fivefold (Ramnarine *et al.*, 1996).

Tiotropium bromide may be anti-inflammatory by acting upon the M₃-receptor as M₃-antagonism leads to a significant decrease in Interleukin (IL)-8 production caused by cigarette smoke Gosens *et al.* (2009). IL-8 is a potent chemotactic mediator that may lead to the prototypically dry cough that smokers get (Jatakanon *et al.*, 1999). Another proposed anti-inflammatory mechanism is that antimuscarinics can prevent neutrophil infiltration. Zhang *et al.* (2010) demonstrated that Matrix Metalloproteinase Type 9 (MMP-9) is up-regulated by cadmium found in cigarette smoke. MMP-9 is a potent mediator of chemotaxis and is an enzyme that degrades Type IV and V collagen; allowing leukocytes to infiltrate into damaged tissue. Tiotropium bromide at picomole concentrations significantly reduced the expression of mRNA of MMP-2, MMP-7 and MMP-9 by 40% (Asano *et al.*, 2008). Consistent with this observation, another group has demonstrated that tiotropium bromide reduces a broad number of inflammatory mediators (IL-6, Keratinocyte-derived Chemokine (KC), Tumour Necrosis Factor (TNF) α , Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Protein (MIP)-1 α , and MIP-2)

released by phagocytic cells (Wollin and Pieper, 2010) and this may be attributable to reduction in the number of phagocytes recruited to the airways.

Birrell *et al.* (2014), recently, demonstrated that tiotropium bromide at 10 $\mu\text{g ml}^{-1}$ and 100 $\mu\text{g ml}^{-1}$ could significantly reduce the cough response of guinea pigs when challenged with aerosolised capsaicin. Further, tiotropium bromide and, the structurally similar, ipratropium bromide inhibited vagal nerve depolarisations by around 60%, but this effect was not demonstrated by two other anticholinergics; glycopyrrolate or atropine. Atropine, glycopyrrolate, ipratropium bromide and tiotropium bromide all have similar broad activity at the various muscarinic receptors subtypes (M_1 , M_2 , M_3) (Haddad *et al.*, 1999; Barnes, 2000), although do have some differences in pharmacokinetics (Barnes, 2000). It is suggested therefore that tiotropium bromide may have other pharmacological activities beyond muscarine receptor antagonism exhibited by glycopyrrolate or atropine such as an ability to act on TRPV1 receptors.

Our study is primarily concerned with the purported antitussive action of tiotropium bromide and in turn two tiotropium bromide analogues, CHF-5843 and CHF-6023 and whether these drugs have antitussive activity in an ozone sensitised hypertussive model of cough.

1.5.3 Beta-adrenergic agonists

Bronchodilators such as the β_2 -agonists were hypothesised to act as antitussives. The proposal was that deforming the airways through changes in smooth muscle tone during bronchoconstriction excited airway mechanoreceptors and could lead to a cough response (Karlsson *et al.*, 1999). A

bronchodilator therefore would oppose this method of eliciting a cough. Isoprenaline (a selective β -agonist) can suppress citric acid induced cough and bronchoconstriction in asthmatics (Karlsson *et al.*, 1999), but β_2 -agonists do not suppress citric acid induced cough in healthy humans (Pounsford *et al.*, 1985). It is unlikely that bronchodilators are true antitussives, although they may alleviate a symptom that leads to cough.

The relevance to our study is that to control for the primary efficacy of anticholinergics, bronchodilation, β_2 -agonists act as a bronchodilator but via stimulating the sympathetic nervous system rather than by inhibiting the parasympathetic nervous system. This allows us to control for parasympathetic effects on cough, if any, and the effect on bronchotone simultaneously.

1.5.4 Roflumilast, a PDE 4 inhibitor

Roflumilast is a selective Phosphodiesterase (PDE) inhibitor particularly important in the treatment of COPD as it helps mitigate multiple features of the aetiology. PDE inhibition directly reduces inflammation and as a consequence has positive therapeutic outcomes on mucociliary malfunction, lung fibrosis, emphysematous remodelling, pulmonary vascular remodelling and pulmonary hypertension (Hatzelmann *et al.*, 2010). Roflumilast blocks the metabolism of cAMP, enhancing cAMP levels which in turn significantly augments IL-10 production and leads to subsequent depression of the release of TNF- α and IL-6 (Kambayashi *et al.*, 1995; Page and Spina, 2011). Reduction of TNF- α and IL-6 leads to a broad decrease in inflammation because it limits the activation of the macrophage-mediated innate immune system (Kambayashi *et al.*, 1995).

Inflammation sensitises the cough reflex and this is a mechanism of action by which cough sensitization in the ovalbumin-sensitised guinea pig models occurs (Liu *et al.*, 2001; Reynolds *et al.*, 2004). Correspondingly, PDE4 inhibitors can significantly inhibit the citric acid induced cough response in ovalbumin-sensitised guinea pigs (Hj *et al.*, 2004). Interestingly, PDE inhibitors also reduced citric acid induced cough in healthy guinea pigs as well as allergic guinea pigs. PDE1, PDE3 and PDE4 inhibition significantly reduced citric acid induced cough (Mokry and Nosalova, 2011) and this is both an indication that inflammation is an important component of cough sensitization and that inflammation plays a role in both sensitised and healthy guinea pigs. In humans, cilostazol, a PDE3 inhibitor, has demonstrated some efficacy against capsaicin-induced cough in humans (Ishiura *et al.*, 2005)

The relevance of roflumilast to our study is that anticholinergics may be anti-inflammatory so roflumilast is a suitable control drug for anti-inflammatory activity. Roflumilast can specifically reduce the release of TNF- α and IL-6, powerful chemokines that signal the innate immune system and act as a control for anti-inflammatory effects on cough. In addition, roflumilast has demonstrated efficacy against cough in allergic guinea pigs and thus provides a useful comparison between guinea pigs and rabbit responses.

1.6 Animal models of cough

Most studies in cough are carried out *in vivo*, although *in vitro* models, particularly single fibre and patch-clamped ion channel recordings, supplement

the primary *in vivo* models (Mackenzie *et al.*, 2004). Many of the commonly used research animals have been studied and are discussed below with the majority of preclinical studies using guinea-pigs, rats, rabbits, cats and dogs (Belvisi and Bolser, 2002).

1.6.1 Mice, Ferrets and Rats

It is commonly believed that mice do not cough since they lack the necessary neurosensory reflex apparatus (Karlsson *et al.*, 1988). In mice, ferrets and rats, the physiological response of cough is not observed, although they do appear to be adequately served by an expiration reflex rather than a cough (Korpás and Kalocsayová, 1975). However, mice have been useful for elucidating sensory afferent pathways in the airway (Carr and Undem, 2003) and this is partly because the mouse genome is known. Ferrets also lack the cough reflex and while rats do cough they respond, unusually, with hyperpnea and deep breaths (Widdicombe, 1996b).

1.6.2 Horses and Pigs

Horses are not generally used in primary research, but cough is considered “the most reliable non-invasive indicators of airway disease in horses” (Duz *et al.*, 2010) and so studies have been undertaken to investigate cough in this species. The pig has also been studied in a similar fashion to the horse and for similar reasons as cough is also considered a key biomarker of respiratory disease in this species (Ferrari *et al.*, 2008).

1.6.3 Cats

Early cough studies done were almost entirely based on investigations in the anaesthetised cat (Widdicombe, 1954) and the cat model is the predominant model in the neurophysiology of the cough reflex (Fox *et al.*, 1993; McLeod *et al.*, 2008; Baekey *et al.*, 2003; Mutolo *et al.*, 2008). Few provoked cough models appear to exist, but cats have been used in the study of GABA-B antagonists (Bolser *et al.*, 1993), Neurokinin Receptor Type 1 (NK₁) receptor and NK₂ receptor antagonists and their corresponding role of these receptors in cough (Bolser *et al.*, 1997).

1.6.4 Dogs

Few groups appear to have used the dog as a model for cough research, although dogs responded to citric acid (1%) and coughed around 1 to 10 times within a 10 minute period (Jackson, 1988). The dogs could be repeatedly dosed every hour and responded similarly on each occasion (Jackson, 1988; Jackson *et al.*, 1989). House *et al.* (2004) have demonstrated that dogs can be allergen sensitised to induce cough by neonatally exposing dogs to ragweed. In their study, the cough response increased (both frequency of cough and the magnitude of the response) as well increasing respiratory rate, Total lung resistance (R_L) and decreasing Dynamic lung compliance (C_{dyn}). Recently, GSK administered GSK2339345 (a Na⁺ channel blocker) and citric acid intratracheally, via a percutaneous catheter, to conscious dogs and demonstrated an extremely effective response, observing some 125 coughs

within 10 minutes (Kwong *et al.*, 2013).

1.6.5 Non-Human Primates

Primate cough studies are not routinely conducted, but are of great interest owing to the closeness of primates to human phylogeny (Mackenzie *et al.*, 2004). Primates cough in response to citric acid exposure and that cough response can be sensitised by successive exposure to house dust mite allergen (Schelegle *et al.*, 2001). Primates have also been used for studies in cough modulation and neoplasticity in the nTS and thus primate responses are considered particularly relevant to humans (Chen *et al.*, 2003; Joad *et al.*, 2007).

1.6.6 Guinea-pigs

Guinea pigs are considered to be the “gold standard” animal model in cough research (Morice *et al.*, 2007) and they have been extensively studied, both *in vitro* and *in vivo* with a range of mechanical induction, chemical stimuli and antitussive agents. Guinea pigs are desirable as an animal model of cough because they are inexpensive, small, very sensitive to tussive stimuli and with a similar respiratory physiology to man (Mackenzie *et al.*, 2004).

Guinea pigs cough to many different tussive stimuli such as citric acid, capsaicin and resiniferatoxin. This response has been compared to the response in man (Laude *et al.*, 1993) and, it is of note, that citric acid provocation is the most common tussive agent used in both guinea pig and man (Mackenzie *et al.*, 2004). The allergic guinea pig model, a model used in the study of asthma and

cough, is a popular model (Bolser *et al.*, 1995; Liu *et al.*, 2001; McLeod *et al.*, 2006; Nishitsuji *et al.*, 2008). Guinea pigs can become allergic to ovalbumin and this can be achieved by exposing guinea pigs to ovalbumin. Guinea pigs exposed to ovalbumin on day 1 and 7 of an experimental schedule demonstrate a hyperallergenic response around day 27 (McLeod *et al.*, 2006). The allergic guinea pig model is particularly useful because it encompasses many of the concomitant conditions that produce a hypertussive cough in man such as hypersecretion of mucus and a pro-inflammatory environment in the lung (Bolser *et al.*, 1995). Allergic models also involve the shedding of airway epithelia which exposes nerves to stimuli in the airway and leads to the proliferation of the nerves in the airway epithelia (Widdicombe, 1995). The guinea pig is also a popular model for the effects of tobacco smoke (Lewis *et al.*, 2007). Tobacco smoke significantly sensitizes guinea pigs to both citric acid and capsaicin induced cough between a day after the initial exposure to tobacco smoke (1 - 5 research cigarettes, 2R4F research cigarettes) with a peak response observed at 3 days post-exposure from (Lewis *et al.*, 2007). Repeated tobacco smoke exposure can potentiate the response; repeated exposure, once a day for 10 days, significantly increased the cough response to citric acid and capsaicin when compared with repeated exposure once a day for 3 days (Lewis *et al.*, 2007).

While of great utility, the guinea pig model has several disadvantages, the most serious of which is that the guinea pig model has had a tendency to identify “false positives” (Mackenzie *et al.*, 2004) that failed to predict early-phase clinical failures of NK₁ receptor (Hill, 2000) antagonists, NK₂ receptor antagonists (Girard *et al.*, 1995) and β_2 -agonists (Karlsson *et al.*, 1999).

These clinical failures may be because the guinea-pig has an especially high density of tachykinin-containing airway C-fibres (Mazzone *et al.*, 2005) that on activation release substance P, an extremely potent inflammatory mediator and bronchoconstrictor agent (Sekizawa *et al.*, 1996). Guinea pig models may be overly sensitive to tussive stimuli and this exaggeration may be what distorts the translation of antitussive pharmacology from guinea pigs to man.

1.6.7 Rabbits

Rabbits are not as common used as guinea pigs as antitussive models for a number of reasons; they are larger, more expensive and do not respond well on their initial “naïve ” dose to citric acid (Adcock *et al.*, 2003). Rabbits do, however, respond to several tussive stimuli such as citric acid (Adcock *et al.*, 2003) and capsaicin (Tatar *et al.*, 1996) *after* they have been sensitised with ozone. Tatar *et al.* (1996) have tended to use rabbits as a comparative species to guinea pigs (Tatar *et al.*, 1996) and of particular note is that this study highlights how poorly healthy rabbits respond to cough stimuli. However, this behaviour is closer to how humans respond to capsaicin and therefore may be a better species to investigate cough responses than guinea pigs (Reynolds *et al.*, 2004) particularly since following exposure to ozone, rabbits exhibit a profound hypertussive state which could provide a novel method of studying cough and antitussives *in vivo* (Adcock *et al.*, 2003).

1.7 Cross-over experimental designs

A cross-over design is an experimental design in which individual subjects receive repeat measures of different treatments during different periods; the subject “crosses over” from one treatment to the next. Many clinical trials employ crossover designs (Wellek and Blettner, 2012) and with relevance to our study they are routinely used in clinical trials involving novel antitussives (GSK, 2014; Khalid *et al.*, 2014; Abdulqawi *et al.*, 2014).

Choosing to use a cross-over design is usually predicated on two key features of this experimental design; the efficient use of subjects to compare treatments and individual subjects acting as their own matched controls. The use of subjects is “efficient” because fewer subjects are needed to reach statistical power and this is because subjects are represented in multiple treatment groups. Subjects are matched controls because each subject is in the control group so their control responses can be matched to their treatment responses and this helps control for subject bias on the dependent variable (Piantadosi, 2013). Firstly, and with relevance to this thesis, cough responses are idiosyncratic and are typified by the wide variance in cough response within a patient group (Khalid *et al.*, 2014; Abdulqawi *et al.*, 2014) so it is useful, therefore, to control for these effects using a cross-over design. Secondly, reducing the number of animals required for an appropriately powered experiment either allows fewer animals to be used, implicitly specified in law (Government, 1986) and desired by the 3R principles, or for a greater breadth of experiments to be undertaken with a given number of animals.

Cross-over designs do, however, present a number of confounding statistical variables of which the carryover effect and period effects are of primary concern; both variables reflect the assumption that each trial within an experiment should be statistically independent (Jones and Kenward, 2003; Piantadosi, 2013). Carryover effect is when a treatment affects the successive treatment that a subject receives, it is the compound effect of multiple treatments of one on another, if there is any effect at all. Period effects are where the dependent variable is affected by the number of times an experiment is performed such as tachyphylaxis in response to repeat dosing. Carryover effect can be controlled for, experimentally, by ensuring that the drug washout period is sufficient and ordinarily this defined as a multiple of the drug's half-life (Piantadosi, 2013). Period effects can be controlled for, experimentally, by randomly assigning subjects to a particular sequence of treatments and ensuring that each sequence is sufficiently powered.

Assuming that the experimental design is balanced, the effects of carryover and period can be detected statistically in a robust manner as part of mixed effect modelling. "Balanced" means that each treatment should appear the same number of times within each sequence ("uniform" within sequences) and each treatment appears the same number of times within each period ("uniform" within periods). Mixed effect modelling enables the hypothesis to be tested which in this instance is that the dependent variable (i.e. cough response) is entirely explained by the independent variable (i.e. treatment group) and any compounding variable (i.e. period or carryover) that significantly correlates with the model may be a covariate of the dependent-independent variable

relationship. In practice, this is achieved by constructing a vector for the carryover and the period and fitting these against the relationship between the dependent (i.e. cough response) and the independent (i.e. treatment group) and observing whether the inclusion of this variable significantly correlates with the dependent variable.

In this thesis, cross-over designs have been used for a few reasons. Firstly, cough variability was high within subjects during pilot experiments so by individually controlling each subject's responses we can control for this variability. Secondly, there were practical limits on the number of animals that could be used and the amount of time that could be dedicated to this thesis; cross-over design were used to improve the scope of what this thesis could research. Thirdly, cross-over designs are not popular within preclinical antitussive research, but are very popular in clinical antitussive research so this was considered a novel way to translate a commonly used clinical experimental design to preclinical research.

1.8 Aims

A sensitised rabbit model of cough may be a better model to test, screen and validate putative antitussives as it may better reflect the disease state of 'hypertussive' cough. Any novel animal model of cough must be put in context with the current gold standard which in this instance is the *in vivo* guinea pig model of cough. Similarly, however, this must be a comparison between a sensitised guinea pig model and a sensitised rabbit model. The model described

by Adcock *et al.* (2003) will serve as a basis for these investigations and the effect of ozone on the lungs.

Therefore with the aim of establishing and validating a sensitised rabbit model of cough, establishing, validating and comparing this rabbit model of cough to a sensitised guinea pig model of cough and then investigating putative antitussives in the context of these models the objectives of this thesis are:

1. *To attempt to find the best objective method of classifying cough sounds by identifying parameters in the time and frequency domain.*
2. *To establish a normative cough response in the rabbit and investigate whether cough responses can be sensitised following exposure of the lungs to ozone.*
3. *To investigate the effect of bronchodilation on cough responses and lung function in the normal and ozone treated rabbits.*
4. *To investigate the effect of acute inflammation on the cough response in the normal and ozone treated rabbits.*
5. *To establish whether ozone exposure can sensitise guinea pigs to citric acid.*
6. *To validate the effect of various current and putative antitussives in hypertussive cough and compare these response between a sensitised rabbit model and a sensitised guinea pig model.*
7. *To investigate the mechanism of action of ozone on hypertussive cough.*

Chapter 2

Materials and methods

2.1 Animals

Male New Zealand White rabbits (2.5 - 4kg) were supplied from Northwick Park, London, UK. Male Dunkin-Hartley Guinea pigs (300g - 500kg) were supplied by Harlan, Oxfordshire, UK. The animals were housed in the biological services unit of King's College London, with a 16 hour day and 8 hour night cycle. Food and water were accessible ad libitum and routinely checked by the biological science unit's technical staff.

2.2 Materials

The following materials were used during these studies:

- Atropine (Sigma-Aldrich, A0132)
- CHF-5843 (Chiesi Farmaceutici, CHF-005843 Batch 8 2012-05-31)

- CHF-6021 (Chiesi Farmaceutici, CHF-006021 Batch 3 2012-05-30)
- Capsaicin (Sigma-Aldrich, M2028)
- Chlordiazepoxide (Sigma-Aldrich, C2517, Chlordiazepoxide hydrochloride)
- Chlorpheniramine (Sigma-Aldrich, C3025, Chlorpheniramine maleate)
- Citric acid monohydrate (Sigma-Aldrich, C7129, Citric Acid Monohydrate)
- Codeine (Sigma-Aldrich, C5901, Codeine)
- Diphenhydramine (Santa Cruz Biotechnology, SC-204729, Diphenhydramine hydrochloride)
- HC-030031 (Chiesi Farmaceutici, HC030031, 2014-02-10)
- Isoflurane (Sigma Delta)
- Ketamine (Pfizer, Vetalar[®], 100mg/ml, 0.01%)
- Levodropropizine (Eurodrug, The Hague, the Netherlands)
- Lipopolysaccharides from Escherichia coli 0128:B12 (Sigma-Aldrich, L2755)
- Methacholine (Acetyl- β -methylcholine chloride, Sigma-Aldrich, MW 195.69, S/N A2251)
- Pentobarbitone (Merial, Euthanal[®], G00801A, Pentobarbitone 10%)

- Roflumilast (Kemprotec, Roflumilast, CAS 162401-32-3)
- Salbutamol (Sigma-Aldrich, S8260, Salbutamol)
- Solutol (Sigma-Aldrich, Kolliphor® HS 15, 42966)
- Sterile saline (Baxter Healthcare Ltd, 0.9%)
- Theophylline (Santa Cruz Biotechnology, sc-202835A, Theophylline)
- Tiotropium bromide (Chiesi Farmaceutici, CHF-005121 Batch 3 2012-06-11)
- Tween20 (Fisher Scientific, T/4206/60)
- Urethane (Sigma-Aldrich, U2500, 25%)
- Xylazine (Bayer, Rompun®, xylazine 2%, 23.32 mg ml⁻¹)

Citric acid, methacholine, salbutamol, levodropropizine and codeine were all dissolved in sterile saline. Roflumilast, CHF-5843 and CHF-6021 were poorly soluble in saline so were made up in neat solutol (2%) and diluted to their final concentration on the day of use.

Citric acid was prepared to a stock solution of 1.6M for rabbits and 300mM for guinea pigs for each batch of animals and diluted on the day of use. Methacholine was made up to a stock solution of 80mg ml⁻¹ and serially diluted 1:1 with saline on the day of use to obtain the range of doses. Tiotropium bromide was made up to a stock solution of 6mM and diluted with sterile saline on the day of use. Salbutamol, roflumilast, levodropropizine and codeine were all made up on the day of use.

2.3 Plethysmyography

A custom-made plethysmyography chamber was used to expose animals to ozone and administer aerosolised treatments. The custom-made plethysmyography chamber was also used for the cough experiments performed in the rabbit. The chamber was a Perspex box attached to a pressure transducer (Sensor Technic, differential pressure transducer, T-59291) and a microphone (EMKA, electret microphone) that lead into the data acquisition system (EMKA, PZ100W-Z). In addition, a secondary microphone (EMKA, electret microphone) fed into a computer sound card and recorded the raw audio for playback. A programmatically controlled solenoid regulated valve (EMKA, electro-valve) on the top of the box was used to control the flow of nebulised aerosols to the box. The microphone and the pressure transducer that were part of the EMKA system were sampled at 1kHz while the secondary microphone running into the computers sound card was sampled at 44kHz. The experimental setup is illustrated in Figure 2.1.

There was an exhaust hole in the upper-left end of the box, 5mm in diameter to ensure a continuous through flow of the nebulised agent. All inputs and interfaces to the box were secured with rubber bungs to ensure an airtight seal.

Lastly, the chamber was filled 2cm deep with animal bedding to improve the acoustics, make the animal more comfortable in an attempt to keep it as calm as possible and to absorb excretory products from neutralizing or interfering with the delivered agents such as citric acid.

Cough experiments performed in the guinea pigs used a plethysmyography

box manufactured by EMKA. It, similarly, was a Perspex box attached to a pressure transducer (Sensor Technic, differential pressure transducer, T-59291) and a microphone that lead into the data acquisition system (EMKA, PZ100W-Z). A programmatically controlled solenoid regulated valve (EMKA, PZ100W-Z) on the top of the box was used to control the flow of nebulised aerosols to the chamber. The experimental setup is illustrated in Figure 2.2.

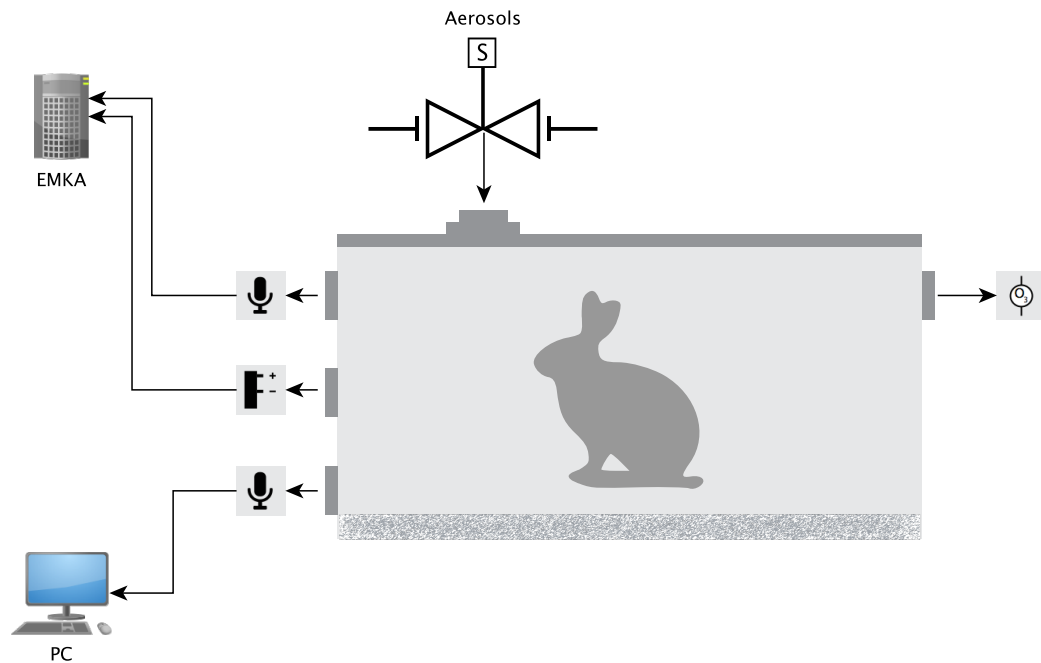


Figure 2.1: A diagram of the custom-made rabbit plethysmyography chamber. Two data acquisition systems on the left-hand side, EMKA and PC, record sound and inter-chamber pressure changes (EMKA) and audio (PC). Aerosols, sensitization agents, treatments or tussives, are introduced via the solenoid regulated valve at the top of the chamber. An exhaust on the right hand side allows air to freely escape the chamber and avoid a build-up of carbon dioxide. The rabbit is sitting on a layer of sawdust bedding.

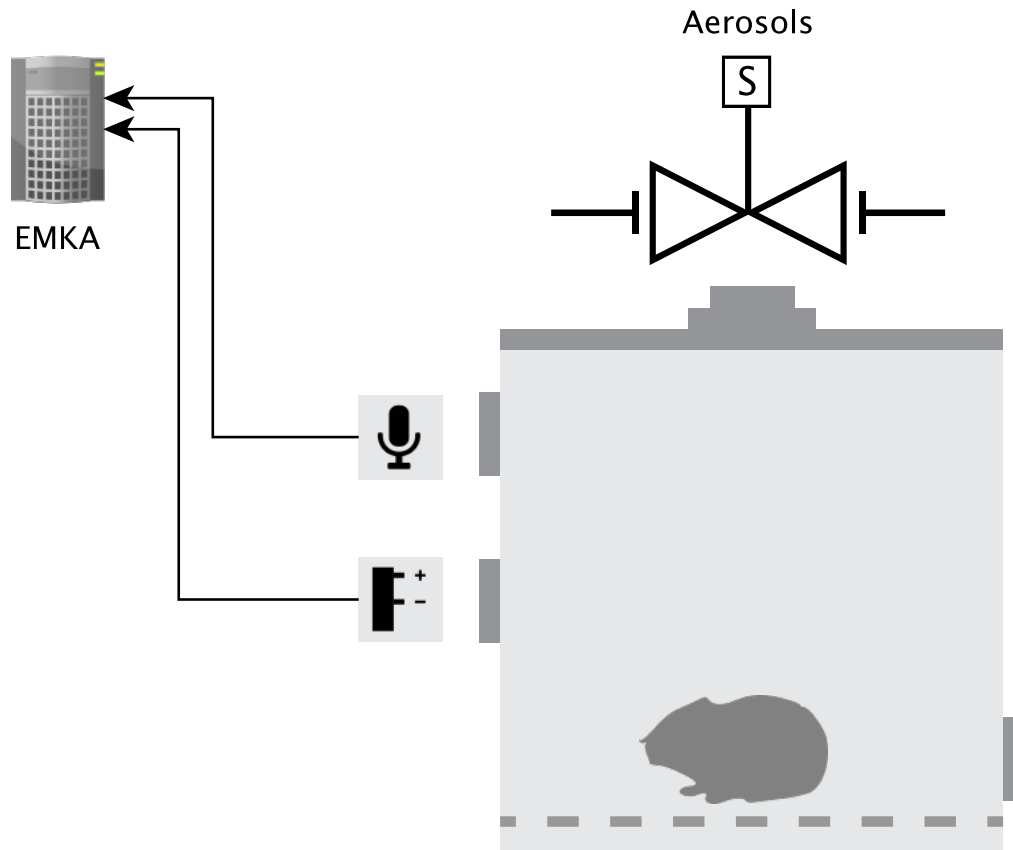


Figure 2.2: A diagram of the EMKA guinea-pig plethysmyography chamber. The acquisition systems is on the left-hand side, recording pressure changes and audio. Aerosols, sensitization agents, treatments or tussives, are introduced via the solenoid regulated valve at the top of the chamber. An exhaust on the right hand side allows air to freely escape the chamber and avoid a build-up of carbon dioxide. The guinea pig is sitting on a perforated plastic layer.

2.4 Citric acid and cinnamaldehyde induced cough

Conscious rabbits were placed in the plethysmyography box (See 2.1) and allowed to acclimatise for 15 minutes. Recording began and for the first three minutes a pre-dose period was recorded which was a negative control to establish that the rabbit was not coughing before being exposed to citric acid. After the first three minutes, the dosing period began, the nebuliser was turned on to maximum output, the solenoid valve on the chambers opened and the air flow was set to 5 Lmin⁻¹ to deliver either aerosolised citric acid or cinnamaldehyde for 10 minutes. After the dosing period, the post-dose period began, the nebuliser was turned off, the solenoid valve closed and the airflow was switched off.

Conscious guinea pigs were placed in the plethysmyography box and the method was identical to the rabbit method with the exception that the citric acid and cinnamaldehyde concentration was allometrically scaled between the rabbits and guinea pigs.

The polyurethane nebuliser cups that held the agents before being aerosolised were dissolved by the cinnamaldehyde at all doses. Thus, a Low Density Polyethylene (LDPE) falcon tube was placed in the nebuliser instead of the normal polyurethane nebuliser cups and held against the ultrasonic vibrating plate with a clamp stand. Cinnamaldehyde was difficult to nebulise so the nebuliser was started 5 minutes before the aerosol was delivered. This was to build up a vapour reservoir of cinnamaldehyde before delivery and to warm the solution slightly.

2.5 Cough counting and profiling

The number of coughs and sneezes were counted during the 10 minute dosing period and the 5 minutes post-dose period.

Coughs made by rabbits and guinea pigs were classified using the cough analyser in the EMKA system. Coughs were classified as the simultaneous increase of the pressure and sound rising sharply above a threshold and back to baseline within 500 ms(see Figure 2.3). The thresholds were determined in initial experiments.

In addition, coughs in the rabbits were classified by listening to the audio recording and subjectively determining that a cough had occurred. Characteristic frequency bands between 3500Hz to 6000Hz in spectrogram associated with the temporal components of the cough (see Figure 2.4) were used to visually assist with seeking through the audio traces for explosive sounds. Coughs events were mapped the audio recording using the open-source Sonic Visualiser software (Cannam *et al.*, 2010), this consisted of annotating the signal by manually drawing a small rectangle around the sound event. These annotations were extracted as an XML file and used to calculate the time interval and the amplitude of the sound event. This was done because I suspected that the EMKA system was falsely identifying all loud events, such as sneezes, as coughs.

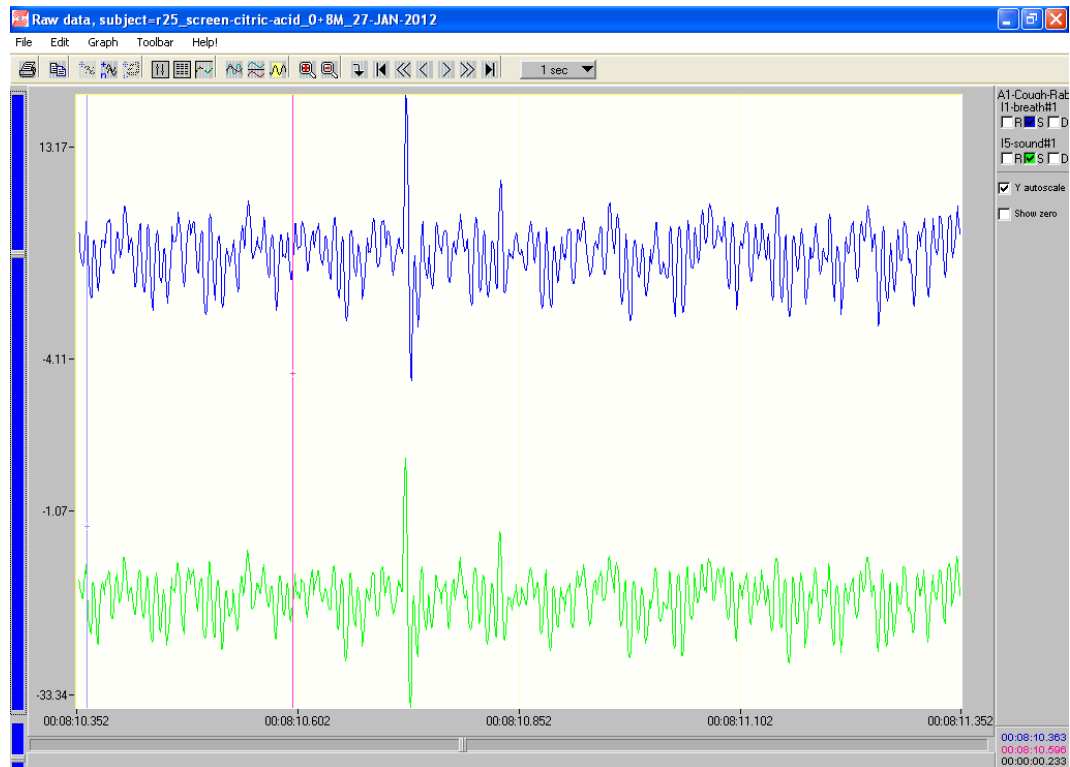


Figure 2.3: A screenshot of the simultaneous change in pressure and sound that EMKA uses to classify a cough event. The cough shown was provoked by exposing a rabbit to aerosolised citric acid.

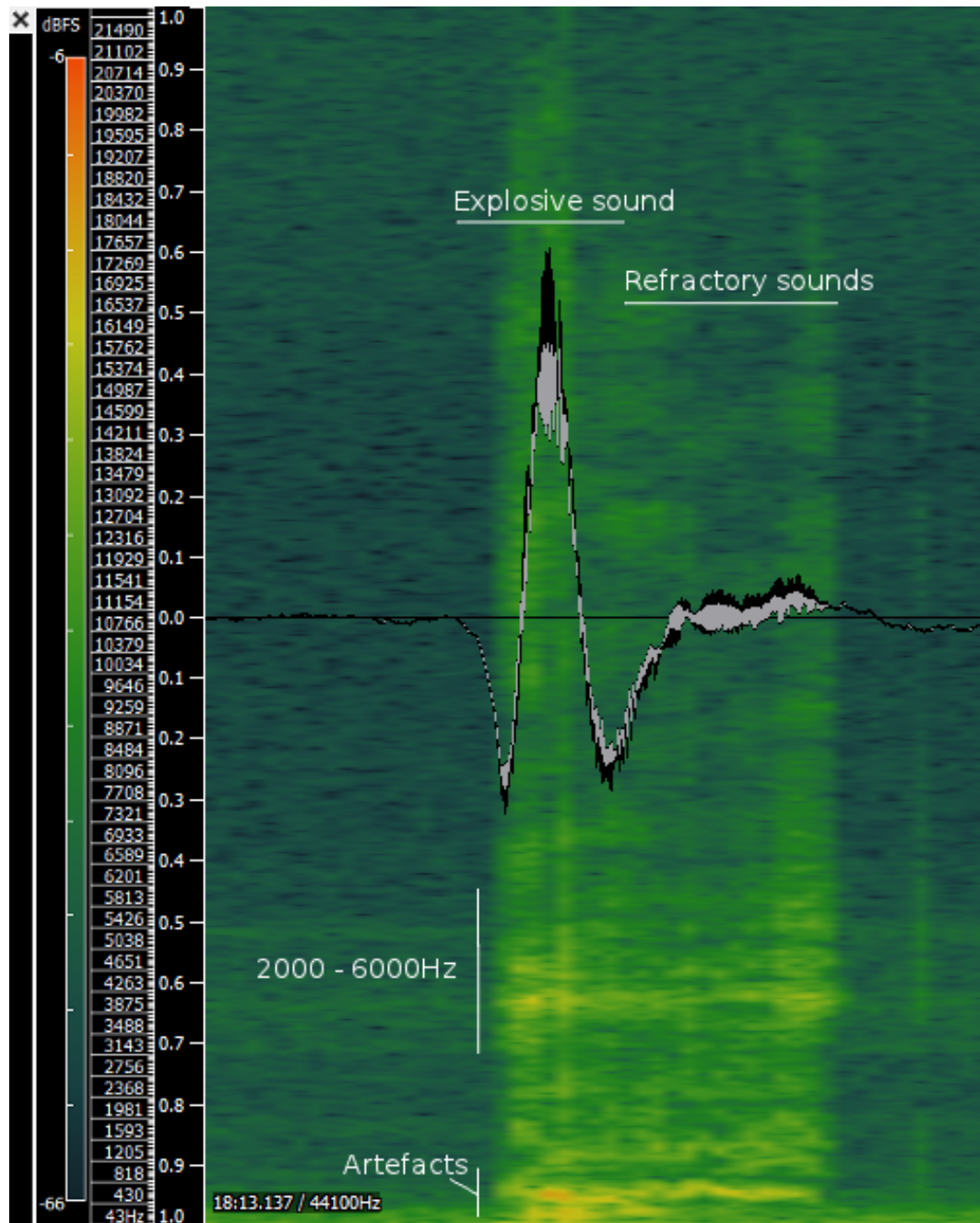


Figure 2.4: A screenshot of Sonic Visualiser and how the audio signal and frequency domain were manually observed to determine a cough. The cough shown was provoked by exposing a rabbit to aerosolised citric acid.

2.6 Anaesthetic regime

Rabbits were anaesthetised with 34 mg kg^{-1} ketamine and 20 mg kg^{-1} xylazine i.m. Rabbits were checked for adequate anaesthesia every 20 minutes and given a further maintenance dose of 50%, if further anaesthesia was required then 25% of the original dose was given every 20 minutes until adequate anaesthesia was obtained. This anaesthetic regime was chosen so that the rabbits could be non-invasively monitored without the need for artificial ventilation in order to monitor dynamic compliance, as opposed to static compliance, and R_L .

Guinea pigs were terminally anaesthetised with urethane (25%) i.p. given in decrementing doses 4 times, every 30 minutes for 2 hours. The dosage started at 2 g kg^{-1} , then to 1 g kg^{-1} ($\frac{1}{2}$ of the original dose) and then 0.5 g kg^{-1} ($\frac{1}{3}$ of the original dose) until adequate anaesthesia was achieved.

Adequate anaesthesia, for both guinea pigs and rabbits, was determined as the abolition of the pain reflex, confirmed by a pinch to the Achilles tendon, or the gag reflex, confirmed by inserting an endotracheal tube.

2.7 Lung function

C_{dyn} and R_L were the two main lung function parameters measured. C_{dyn} is an index of the elastic properties of the airways while R_L describes the mechanical opposition to air flow in the lungs. C_{dyn} is measured in ml/cm H₂O calculated by

dividing the tidal volume by the change in Trans Pulmonary Pressure (TPP):

$$C_{dyn} = \frac{\int_{t_1}^{t_2} V.V\delta t}{\int_{t_1}^{t_2} P.V\delta t} \quad (2.1)$$

R_L is measured in cm H₂O.s.L⁻¹ calculated by dividing the in TPP by the flow:

$$R = \frac{\int_{t_1}^{t_2} P.F\delta t}{\int_{t_1}^{t_2} F.F\delta t} \quad (2.2)$$

Rabbits were anaesthetised according to the standard regime (see section 2.6) and an endotracheal tube (Mallinckrobt I.D. 3.0) was then inserted into the trachea, assisted by holding the tongue up and away to expose the glottis. Once placed, the pilot balloon was inflated and condensate on the endotracheal tube was confirmation that the tube was correctly placed. An oesophageal balloon was inserted down the oesophagus approximately 10cm.

The rabbit was then transferred onto a heating mat (Harvard Homeothermic Blanket) and a temperature probe was inserted into the rectum to thermostatically maintain the temperature of the rabbit at around 37°C. The endotracheal tube was attached to a heated pneumotachometer (Number 11, Fleisch) connected to a pressure transducer (MuMed BR8101, S/N 960303, ± 2cm water) to obtain a measure of airflow. The oesophageal balloon was connected to the negative side of the pressure transducer (MuMed BR8101, S/N 960338, ± 20cm water) to obtain a measure of intrapleural pressure. The positive side of the pressure transducer (MuMed BR8101, S/N 960338, ± 20cm water) was connected to the port of the pneumotachometer proximal to the animal to obtain a measure of mouth pressure. TPP was calculated as the difference

between the mouth and intrapleural pressure.

An online recording of airflow and TPP (Bio-recorder BR8000, Mumed Systems Ltd.) was used to calculate the tidal volume and respiratory rate. TPP, airflow and tidal volume were used to calculate TPP, R_L and C_{dyn} . Observation of an appropriate signal on the airflow and pressure traces confirmed that the balloon and the endotracheal tube were correctly placed.

Baseline variables (R_L , C_{dyn}) were monitored over a 5 to 8 minute period. Rabbits were then exposed for 20 seconds to 0.9% saline by disconnecting the endotracheal tube from the pneumotachograph attaching to tube of the nebuliser (UltraNeb 2000, DeVilbiss Healthcare Ltd.) to deliver aerosols of saline directly to the lung. Rabbits breathed nebuliser solutions spontaneously and a side-arm to the nebuliser tube permitted breathing under atmospheric conditions. The endotracheal tube was reattached and changes in R_L and C_{dyn} were monitored and allowed to reach a steady state. Once this steady-state had been achieved, the endotracheal tube was once again disconnected and a dosing with methacholine was commenced. This cycle was repeated, escalating the dose until a maximum response was achieved. The dose of methacholine delivered ranged from 0.625 mg ml^{-1} to 160 mg ml^{-1} over a period of 20 seconds of exposure. Post-analysis was undertaken to calculate the dose that caused a doubling in either respiratory rate or R_L or a halving in C_{dyn} . The recording software ran continuously throughout the experiment.

Guinea pigs were prepared in a similar way with the exception that the anaesthesia was terminal. To place the endotracheal tube, a tracheal cannula (1.65 mm i.d.) was inserted into the lumen of the cervical trachea through a

tracheostomy and tied in place. The guinea pigs were mechanically ventilated in the supine position by a constant-volume ventilator (Model 683, Harvard Apparatus, Natick, MA) at 8 mgkg⁻¹ tidal volume and a frequency of 60 breaths/min. One jugular vein was cannulated for the intravenous administration of drugs. The dose of methacholine delivered ranged from 80 µg ml⁻¹ to 8000 µg ml⁻¹, over a period of 20 seconds of exposure.

2.8 Bronchoalveolar lavage and cytology

Rabbits were anaesthetised according to the standard regime (section 2.6) and once adequately anaesthetised the rabbit was intubated.

A device was constructed to perform the lavage using a 30ml plastic tube, stoppered with a rubber cork in which the cork had two holes drilled through it. Into the two holes in the rubber cork went two three way taps and the output of one was connected to a vacuum source on the benchtop and the other to a piece of plastic tubing. This plastic tubing, approximately 20cm in length, was inserted down the endotracheal tube to deliver saline for the lavage and recover the lavage fluid from the lung. The fluid was left in the lung for no more than a few seconds and the amount of fluid recovered was noted.

50µL of this solution was fixed with 50µL of Turk's solution (Turk Solution, CAT 109277, Merck KGaA, Darmstadt, Germany). Total cell counts were performed using an aliquot of this solution using a haemocytometer. Two 100µL samples of neat Bronchoalveolar Lavage (BAL) fluid were centrifuged (Cytospin 3 Centrifuge, Thermo Scientific Shandon) onto a glass slide. The slides were left

to dry, fixed (Reastain Quick-Diff Kit, Reagen) and then mounted in DPX. Differential cell counts were performed using confocal microscopy with oil at a 40x magnification.

2.9 Sensitization regimes

2.9.1 Ozone sensitization

Rabbits were placed unrestrained in a purpose-built Perspex chamber and allowed to acclimatize for 15 minutes. After 15 minutes, the rabbits were then exposed to ozone or air for 1 hr. Ozone was generated by passing air through an ozoniser (Certizon C25, Sander) at a flow rate of 5 l/min and the exhaust air from the chamber was measured in real-time using an electronic ozone sensor (Aeroqual 200 series, Aeroqual Ltd). An analogue dial controlled the strength of ozoniser output and this was adjusted, using the electronic sensor as a measure, to achieve a target atmosphere of 2ppm.

Guinea pigs were placed in the same aforementioned chamber, between 2 to 4 at a time, but the exposure time was 30 minutes and the target atmosphere was 2ppm. An unacceptable rate of cyanosis resulted from using the same exposure protocol for both rabbits and guinea pigs so the exposure time was reduced from 1 hour to 30 minutes while maintaining the same target atmosphere.

2.9.2 LPS sensitization

Originally, LPS was given as an aerosol but because I was unsure as to whether LPS was getting delivered into the lung I also tried intratracheal administration. This was based on pilot experiments performed with the first 8 guinea pigs and both methods are stated below.

Aerosol Guinea pigs were placed in a vapour tower (Chisei Farmaceutici, Custom made) and allowed to acclimatise for 15 minutes. After 15 minutes, the guinea pigs were exposed to an aerosol of LPS ($50\mu\text{g ml}^{-1}$) or vehicle (saline). The solution of LPS was made up on the day from a frozen aliquot. The guinea-pigs were then left for 4 hours before further experiments. LPS is a bacterial endotoxin and causes inflammation in the airways when inhaled (Riccio *et al.*, 1997; Brown *et al.*, 2007). 4 hours is a sufficient amount of time for an inflammatory response to manifest and neutrophilia can be seen from 2 hours post insult (Brown *et al.*, 2007).

Intra-tracheal Guinea pigs were anaesthetised with isoflurane (Sigma Delta) from an isoflurane vaporiser (Sigma Delta, Penlon) into a perspex chamber. Once adequate anaesthesia was achieved (see section 2.6), the guinea pig was removed from the perspex chamber and attached to a tilt table (Chisei Farmaceutici, Custom made). The tilt table was adjusted, the guinea pigs mouth opened and a otolaryngoscope was inserted. Once the trachea was located, a tube was inserted into the trachea and 1ml of $50\mu\text{g ml}^{-1}$ LPS was administered. Throughout, anaesthesia was maintained by routinely applying more

anaesthetic using a snout mask. The animal was then placed in a second container and allowed to recover, recovery took around 15 - 20 minutes.

2.9.3 Capsaicin desensitization

Rabbits were treated with capsaicin (total dose of 80mg kg^{-1} , s.c.) administered over a period of 3 days according to Table 2.1 . The rabbits received a pre-medication 15 minutes before each capsaicin injection of theophylline 2mg kg^{-1} , atropine 1.2mg kg^{-1} , diphenhydramine 2.5mg kg^{-1} , and chlordiazepoxide 1.2mg kg^{-1} (i.p.). Capsaicin was then injected 15 minutes after pre-medication. The cough response was assessed by a citric acid induced cough experiment 2 days after the last capsaicin injection.

Table 2.1: The capsaicin treatment regime given daily as a subcutaneous injection to the loose skin around the neck and shoulder area in the rabbit.

Day 1	0.3mg/kg
	0.6mg/kg
	1.5mg/kg
	2.6mg/kg
Day 2	5.0mg/kg
	10.0mg/kg
	15.0mg/kg
	20.0mg/kg
	25mg/kg
Day 3	25mg/kg

2.10 Treatment regimes

2.10.1 Codeine

Codeine, 3mg kg^{-1} i.p., 5 minutes before being exposed to citric acid and both guinea pigs and rabbits received the same dose. Each treatment was given as a maximal single dose based on the dosage regimen previously used by Karlsson *et al.* (1990).

2.10.2 Anticholinergics

Anticholinergic treatments were given as an aerosol for ten minutes in the plethysmyography box two hours before ozone sensitization and three hours before further experiments.

Each treatment was given at a maximal single dose specified by Chiesi Farmaceutici, each concentration was specific to each treatment agent. Tiotropium bromide (CHF-5121) was given at $250\mu\text{M}$, CHF-5843 at 2.5mM and CHF-6021 at 2.5mM . The tiotropium bromide stock was kept at a 6mM concentration. CHF-5843 and CHF-6021 were made up in neat solutol and then diluted with saline so that solutol made up 3% of the resulting preparation. Guinea pigs and rabbits were given the same dose of anticholinergics.

2.10.3 Salbutamol

Salbutamol ($50\mu\text{g ml}^{-1}$ guinea pigs and $100\mu\text{g ml}^{-1}$ for rabbits) was given as an aerosol for 2 minutes in the plethysmyography chamber 5 minutes before citric

acid induced cough in the cough experiments and, similarly, 5 minutes before methacholine induced bronchospasm in the lung function experiments.

Each treatment was given as a maximal single dose at a dose considered to be an EC_{100} dose determined by a lung function experiment. Rabbits and guinea pigs were anaesthetised and then exposed to cumulatively increasing doses of methacholine until there was a doubling in R_L and a halving in C_{dyn} . The dose of salbutamol required to reverse methacholine induced bronchospasm was the dose used.

2.10.4 Roflumilast

Roflumilast, 1mg kg^{-1} , was administered i.p. 30 minutes before the further experiments. Both guinea pigs and rabbits received the same dose. Each treatment was given a single maximal dose based on the dosage used by Mokry *et al.* (2008).

2.10.5 Levodropropizine

Levodropropizine was administered, i.p., 30 minutes before further experiments and only guinea pigs received levodropropizine treatment.

Each treatment was given either as a low dose (10mg kg^{-1}) or as a high dose (30mg kg^{-1}), the dosage was determined by previous experiments conducted by a Post-Doctoral Fellow at King's College London.

2.10.6 Chlorpheniramine

Chlorpheniramine was administered, i.p., 30 minutes before further experiments and only guinea pigs received chlorpheniramine.

Each treatment was given either as a low dose (10mg kg^{-1}) or as a high dose (30mg kg^{-1}), the dosage was determined by previous experiments conducted by a Post-Doctoral Fellow at King's College London.

2.11 Protocols

2.11.1 Establishing the normative cough response and validating the EMKA cough system

12 rabbits and 30 guinea pigs were used to establish a dose response relationship to citric acid in the EMKA cough system using a 2x2 randomised crossover design. Citric acid was delivered either as a 0.4M, 0.8M or 1.6M aerosol for the rabbits and 30mM, 100mM or 300mM aerosol for the guinea pigs (see Section 2.4). 7 days later the experiment was repeated and the animals received another random dose. 4 rabbits and 16 guinea pigs were dosed every day for 5 days with 0.8M (rabbits) or 100mM (guinea pigs) citric acid to establish whether the animals became desensitised with regular daily exposure to citric acid.

During this period, optimizations and modifications were made to the experimental setup.

2.11.2 Investigating the effects of ozone on the cough response, lung function and lung inflammation

16 rabbits and 32 guinea pigs were investigated and every animal was “screened” on day 1; this was the first, naïve, citric acid induced coughing experiment with no sensitization and no treatment. The screen established the baseline sensitivity of the animal to citric acid. On day 7 the animals randomly received ozone sensitization or sham (see section 2.9.1), then on day 14 the animals were crossed over (see fig. 2.5). 4 rabbits were crossed-over again to establish the repeatability of the ozone sensitised response (see fig. 2.6). The interval of 7 days between each experiment was used to avoid any possible potentiation of the ozone sensitised response from chronic exposure.

7 days after the last cough experiments finished, lung function experiments were performed (see section 2.7). On day 1 of lung function experiments rabbits randomly received either vehicle (saline) or ozone sensitization for 1 hour and guinea pigs were randomly divided into two groups one control and one receiving ozone sensitization for 30 minutes. 4 hours later, the lung function experiment began. Animals were anaesthetised and intubated, then methacholine was delivered in successive, increasing doses (see section 2.7) until there was a doubling in R_L and a halving in C_{dyn} . The guinea-pigs were terminated and the lungs were removed for histological analysis. The rabbits were recovered from anaesthesia and underwent a second lung function experiment 3 days later, so that each rabbit was individually controlled. At the conclusion of each lung function experiment, a BAL was performed

(section 2.8) and a total and differential cell count was taken. Finally, the rabbit was terminated, dissected and the lungs removed for histological analysis. See Figure 2.5 for a schematic illustration.

2.11.3 Investigating the effects of LPS on the cough response and validating the Buxco cough system

68 guinea pigs were investigated and of this a total of 16 guinea pigs were used to establish a dose response relationship to citric acid. Guinea pigs received either 30mM, 100mM or 300mM as an aerosol in a citric acid induced coughing experiment (see section 2.4) then 7 days later the experiment was repeated. The guinea pigs were then terminated.

52 guinea pigs were sensitised to LPS. Initially, 16 guinea pigs were sensitised to LPS using the aerosol method but no observable signs of inflammation nor any change to the cough response therefore the intra-tracheal method was used for the other 36 guinea pigs (see (section 2.9.2)). Each of the 36 guinea pigs were randomly assigned to receive LPS or vehicle and received either 150mM citric acid or 300mM, divided equally into four groups. See Figure 2.7 for a schematic illustration.

2.11.4 Evaluating the antitussive effects of tiotropium bromide, CHF-5843 and CHF-6021

12 rabbits and 16 guinea pigs received tiotropium bromide treatment, with and without ozone sensitization to establish the effect of tiotropium bromide on the

normotussive and hypertussive cough using the EMKA cough system. Similarly, 8 rabbits received CHF-5843 treatment and 8 rabbits received CHF-6021 treatment, although these drugs were not evaluated in guinea pigs.

7 days after the cough experiments finished the rabbits were used for lung function experiments to establish the effect of tiotropium and the CHF compounds on methacholine induced bronchospasm in exactly the same protocol that was used to assess the effect of ozone on lung function (see section 2.11.2) but animals received tiotropium bromide, CHF-5843 or CHF-6021 treatment before ozone sensitization. Similarly, at the conclusion of the lung function experiments a BAL was performed and a total and differential cell count was taken. Finally, the rabbit was killed, dissected and the lungs removed for histological analysis. See Figure 2.8 for a schematic illustration.

In addition, a further 96 guinea pigs received tiotropium bromide treatment, with and without LPS sensitization to establish the effect of tiotropium bromide on the normotussive and hypertussive cough in the Buxco cough system.

2.11.5 Validating current and testing putative antitussives

8 rabbits and 32 guinea pigs were used in a randomised cross-over design to evaluate salbutamol, roflumilast and codeine. On the first day the animals were screened, this was a negative control for citric acid at a Least Effective Dose (LED). On day 7, the animals received ozone sensitization (positive control) or sham (negative control for ozone) and on day 4 crossed-over. On day 21 the animal received either salbutamol, roflumilast or codeine treatment (see section 2.10) before a citric acid induced coughing experiment. On day 28 and

35 the animals were crossed over with each treatment groups so that each animal received each treatment.

7 days after the cough experiments finished, half of the rabbits were used to validate the dose of salbutamol lung function experiments and half were used to validate the dose of roflumilast. 4 rabbits and 8 guinea pigs were used to validate the dose of salbutamol. Animals were anaesthetised and intubated, then methacholine was delivered in successive, increasing doses (see section 2.7) until there was a doubling in R_L and a halving in C_{dyn} . At this point, salbutamol was delivered as an aerosol, $50\mu\text{g ml}^{-1}$ guinea pigs and $100\mu\text{g ml}^{-1}$ for rabbits, for 2 mins and then the data acquisition was reconnected for 1 minute. The animals were repeatedly dosed with the same dose until the C_{dyn} and R_L had returned to baseline values. 4 rabbits and 8 guinea pigs were used to validate the dose of roflumilast. Guinea pigs were treated with roflumilast (see section 2.10) and half randomly received ozone sensitization while the other half received sham sensitization. Rabbits were treated with roflumilast and received either ozone sensitization or sham and then crossed-over three days later. See Figure 2.9 for a schematic illustration.

In a separate experiment, 32 guinea pigs were used in a randomised cross-over design to evaluate levodropropizine, chlorpheniramine and levodropropizine with chlorpheniramine. On day 1 the guinea pigs were screened, on day 7 they randomly received levodropropizine, chlorpheniramine or levodropropizine with chlorpheniramine treatment (see section 2.10) and on days 14, 21 and 28 they were randomly crossed-over until each subject had received each treatment. Levodropropizine and chlorpheniramine were given

randomly as either a high or low dose.

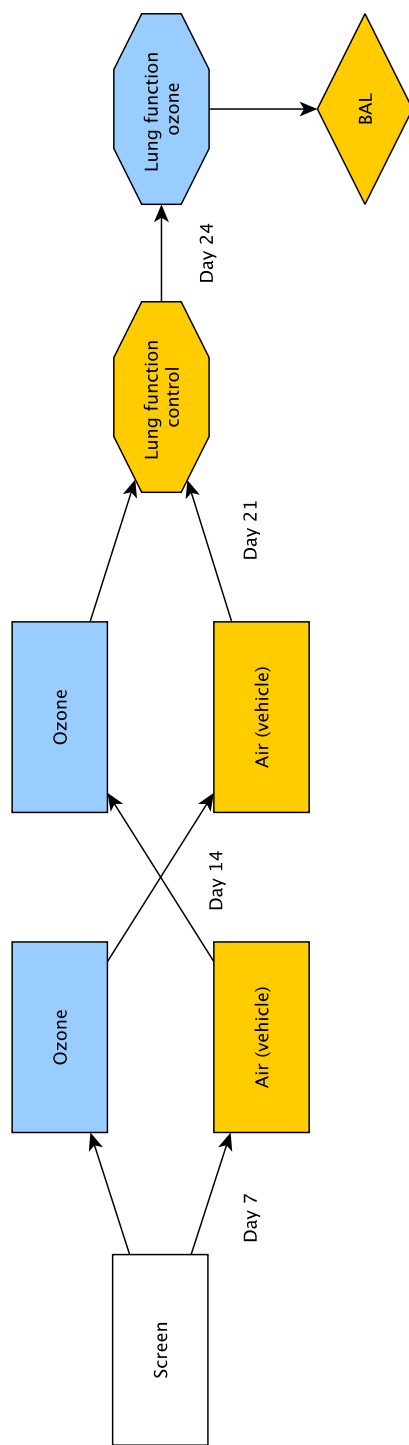


Figure 2.5: Schematic representation of the protocol for investigating the effects of ozone on the cough response using a double-crossover. 16 rabbits and 32 guinea pigs received the single crossover design

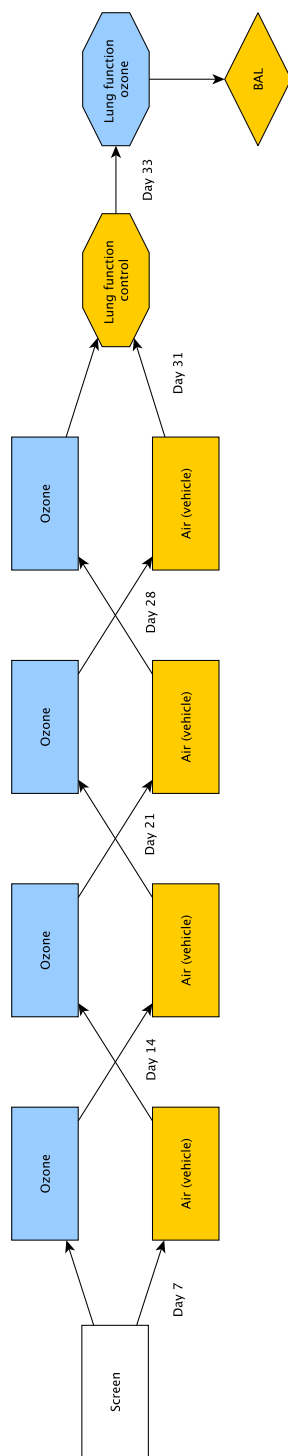


Figure 2.6: Schematic representation of the protocol for investigating the effects of ozone on the cough response using a double-crossover. 4 rabbits received the double crossover design.

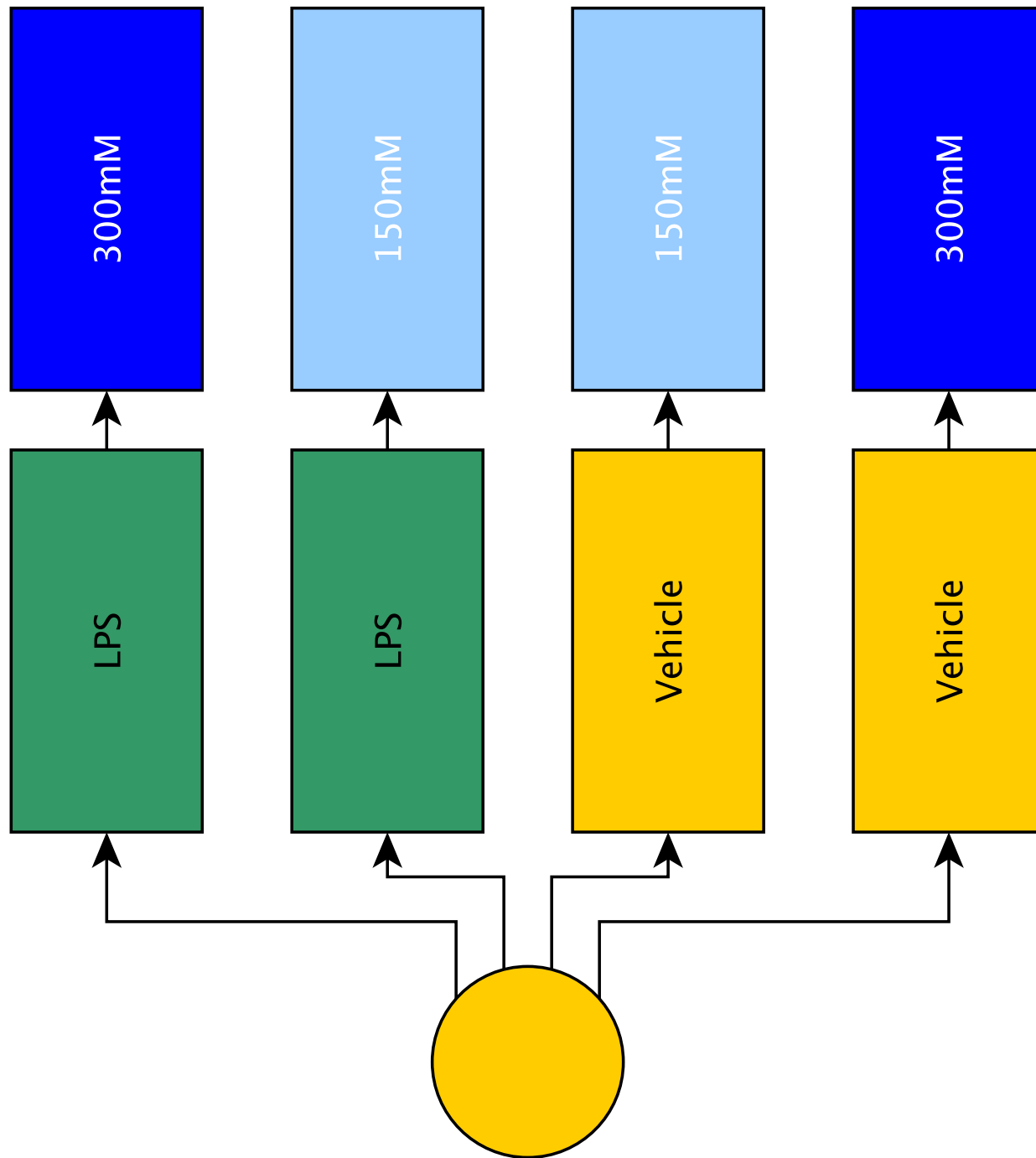


Figure 2.7: Schematic representation of the protocol for establishing the effect of LPS on the citric acid dose response curve in guinea pigs.

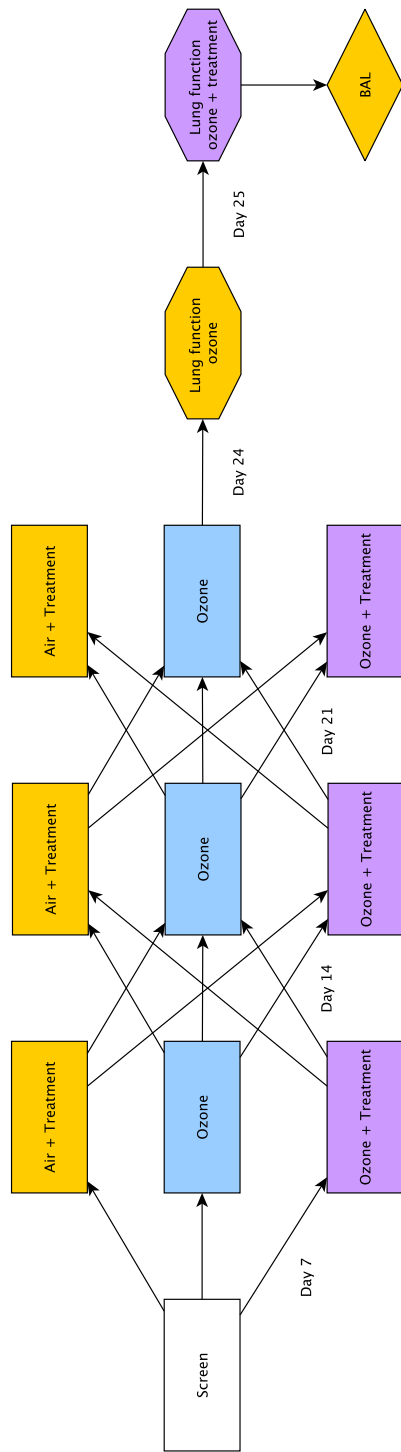


Figure 2.8: Schematic representation of the protocol for evaluating the antitussive effects of anticholinergic treatment on the ozone sensitised citric acid induced cough response in guinea pigs and rabbits

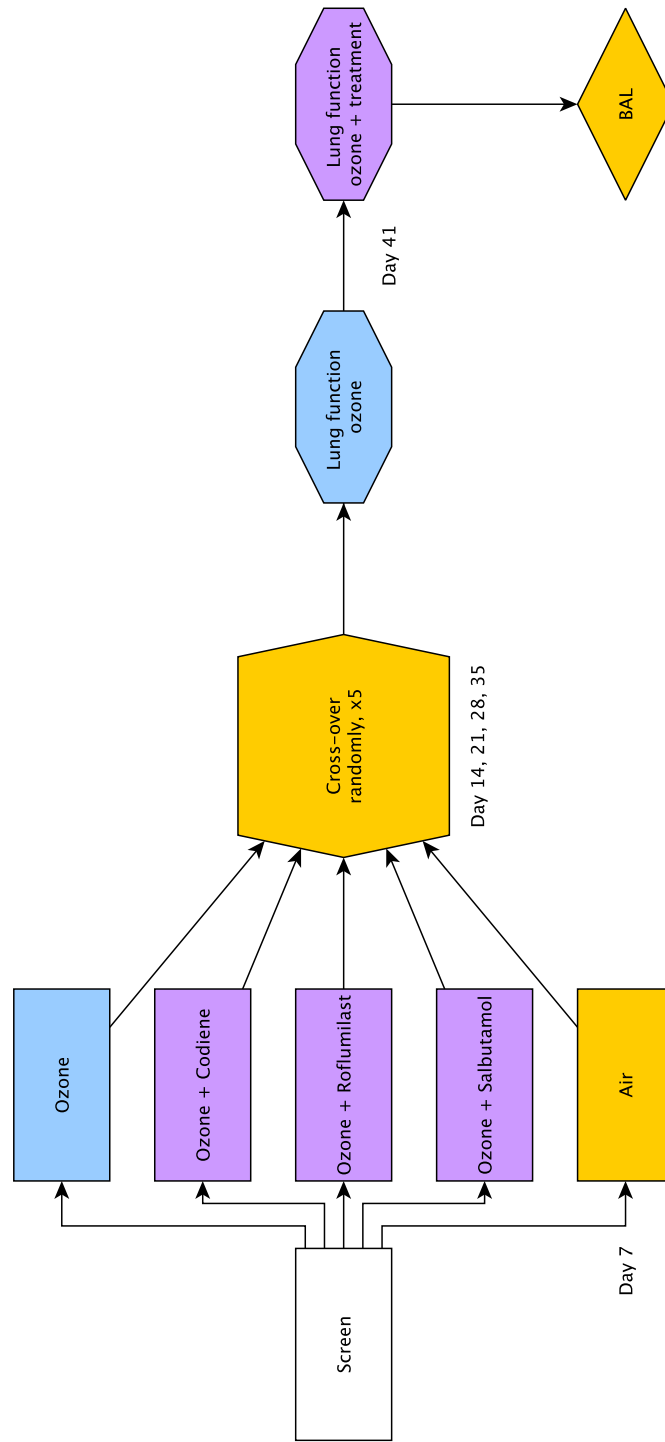


Figure 2.9: Schematic representation of the protocol for validating and testing the effect of putative antitussives on ozone sensitised citric acid cough responses in guinea pigs and rabbits

2.12 Statistical analyses

To classify audio events such as cough and sneeze events, Sonic Visualiser (Sonic Visualiser Copyright © 2005–2012 Chris Cannam and Queen Mary University, London) was used to listen to each individual event and each event was then annotated as a cough or a sneeze. The signal was annotated using Sonic Visualiser by manually drawing a small rectangle around each sound event on an annotations layer within Sonic Visualiser; this meant that the start, end, peak and trough of the event were measured. These measurements were held in an annotation layer, in an XML format.

The annotation layers were analyzed using the Python programming language to script routines to calculate parameters. The width or the interval of an audio event was the difference between the end frame and start frame of an interval and standardized to seconds, the height was the difference between the absolute peak and the absolute trough and standardized to volts. The power of the sound event was calculated in a facile manner by multiplying the width of event by the height of the event.

The response to methacholine was expressed as a percent increase (R_L) or decrease (C_{dyn}) of the post saline values. The Percent Change (PC) R_L and PC C_{dyn} was interpolated from the response versus concentration curve, a Concentration of agonist in mg/ml required to reduce the dynamic compliance 35% from the baseline (PC₃₅) for C_{dyn} and a Concentration of agonist in mg/ml required to reduce the dynamic compliance 50% from the baseline (PC₅₀) for R_L was considered the threshold endpoint.

Data is commonly expressed in graphs as the mean \pm SEM and in the body of the text as the mean \pm 95% Confidence Interval (CI), 3 significant figures are used to represent each datum. For each pairwise comparison, a paired Student's t-test was used and a p-value < 0.05 was considered significant. ANOVA was used when more than one group was compared and D'Agostino's K-squared test for normality was used to determine which post-hoc analysis to perform after an ANOVA.

Repeat measures ANOVA was used when animals received multiple doses and for all cross-over designs. A test for sphericity was performed to control for homoscedacity and, in addition, an a priori Geisser-Greenhouse correction was made to increase the certainty that sphericity had not been violated. A post-hoc Dunnett's multiple comparisons test was conducted to test for pairwise significance of treatment groups relative to the control group; the control group in most cases was the ozone sensitised group.

In addition, for cross-over designs that involved a drug treatment a mixed effect model was performed before the repeat measures ANOVA to determine whether interactions between the treatment (random), sequence (random) or period (fixed) caused confounding carryover effects on the cough response. The expectation-maximization algorithm was used to fit the parameters of the model. In addition, the treatment groups were balanced to control for the effect of first-order carryover effects and subjects were randomly assigned a sequence using a random number generator. The mixed effect model was calculated using the MixedLM function from the statsmodels package in the Python programming language. The parameters for the MixedLM functions were

inputted such that cough, the dependent variable, was grouped by the treatment received and interactions between cough and sequence and cough and period were calculated.

Chapter 3

Results

3.1 The normative cough response and the objective measurement of cough

3.1.1 Parameters and features of cough

Rabbits cough *and* sneeze when exposed to citric acid and cough more than they sneeze with a ratio of 1.72 (\pm 1.18 - 2.26, CI) coughs to 1 sneeze and because they both occur so frequently both were considered when discriminating a cough from a sneeze (Figure 3.1).

Both the cough and sneeze events are very similar and are typified by an abrupt violent sound followed, in some cases, but not all, by refractory sounds. The explosive sound is audibly distinct from many other sounds that the rabbit makes such as scratching, wretching and wheezing. The refractory sounds can vary greatly and there isn't a clear pattern common to either a cough or a sneeze.

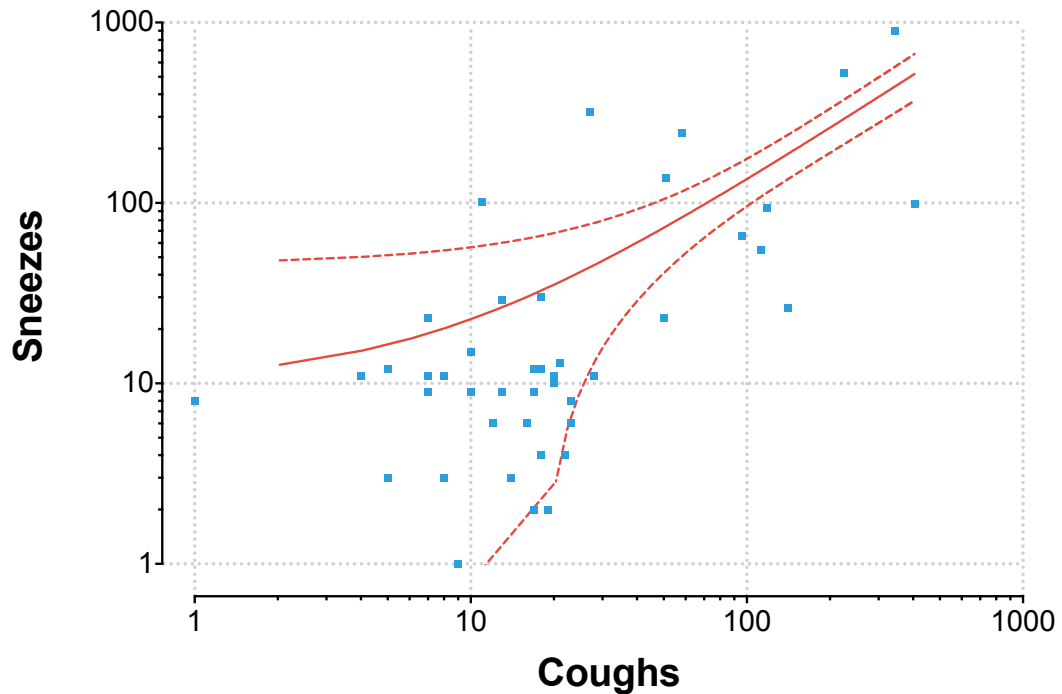


Figure 3.1: The correlation of coughs and sneezes for all rabbits on their first exposure to citric acid. The aerosol concentration of citric acid was either 0.4M, 0.8M or 1.6M. The solid line is an ordinary-least squares linear regression and the dashed line represents the 95% confidence intervals of that regression. n of 60.

Distinguishing the sound of a cough from a sneeze in rabbits based on how the signals change in the time-domain is difficult and error prone, necessitating listening to each cough and sneeze event and manually classifying them. Figure 3.3 and fig. 3.4 are visual illustrations of a typical sneeze and a typical cough and while differences may be observable they are both very similar and this similarity varies depending on whether a cough or sneeze is being observed. Further, they share similar frequency components and simply observing the spectrogram is not sufficient to discriminate a cough from a sneeze and there is a band from 2000Hz to 6000Hz that is common to both these sound events. The

sub 200Hz band is noise from the fumehood and there are some higher frequency components that are roughly 100 times quieter than the 2000Hz - 6000Hz band that may be represent aliases or harmonics of the lower frequencies.

To illustrate the similarity of all of the cough (n of 2932) and sneeze (n of 1884) events observed is illustrated in figure 3.2. There is a 97% overlap between coughs and sneezes when width is considered (133 distinct out of 4816) and 99% overlap between coughs and sneezes when amplitude is considered (48 distinct out of 4816).

Individual cases were observed where a cough was not classified at all (Type I error) or mistakenly identified as a sneeze (Type II error). Sneezes could be mistaken for coughs if the sneeze was as loud, and changed the intra-chamber pressure as much as, a cough (fig. 3.5). Coughs that didn't have a large enough expiration weren't classified because they failed to trigger the pressure signal (fig. 3.6). Coughs that were too quiet for the threshold of the classifier weren't classified (fig. 3.7). Lastly, visual inspection as a means of discriminating a cough from a sneeze was error prone evidenced by (fig. 3.8).

Guinea pigs may cough and sneeze, but the two sounds are either too audibly indistinct or we cannot hear the difference between the two. Further, we cannot disseminate a difference in the frequency spectra between the two. Similarly to the rabbit, the cough is a single explosive event but rarely has refractory sounds. In contrast, the guinea pig has a frequency band common to most coughs around 7200 to 9900Hz, this is higher than the rabbits common frequency band (fig. 3.9).

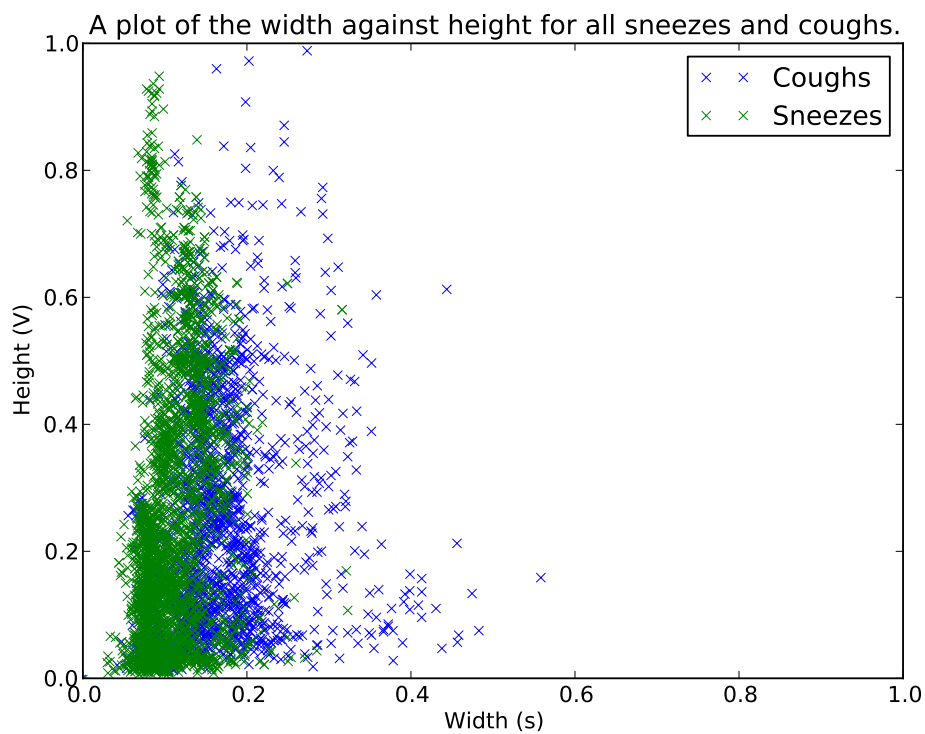


Figure 3.2: The interval width (seconds) of the audio events plotted against the amplitude (V) for all sneeze and all cough events amongst all animals tested.

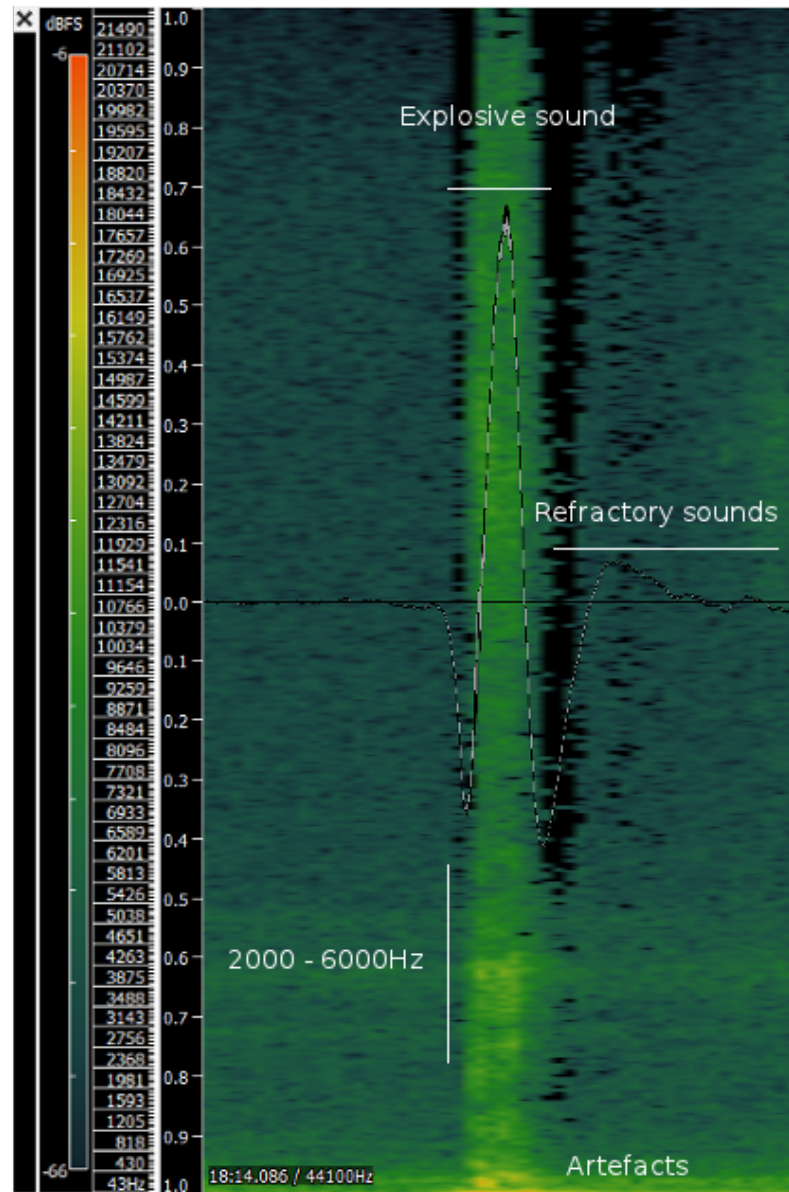


Figure 3.3: A prototypical sneeze in the rabbit. They each consist of a large singular explosive event followed by refractory sound. The explosive phase lasts between 80-500ms. The heat map underneath each audio signal represents the discrete Fourier transform of the audio signal binned at 1024 sample intervals. The region between 2000 and 6000Hz has been highlighted as a region of interest and the region marked artifact indicated noise related to the fume hood.

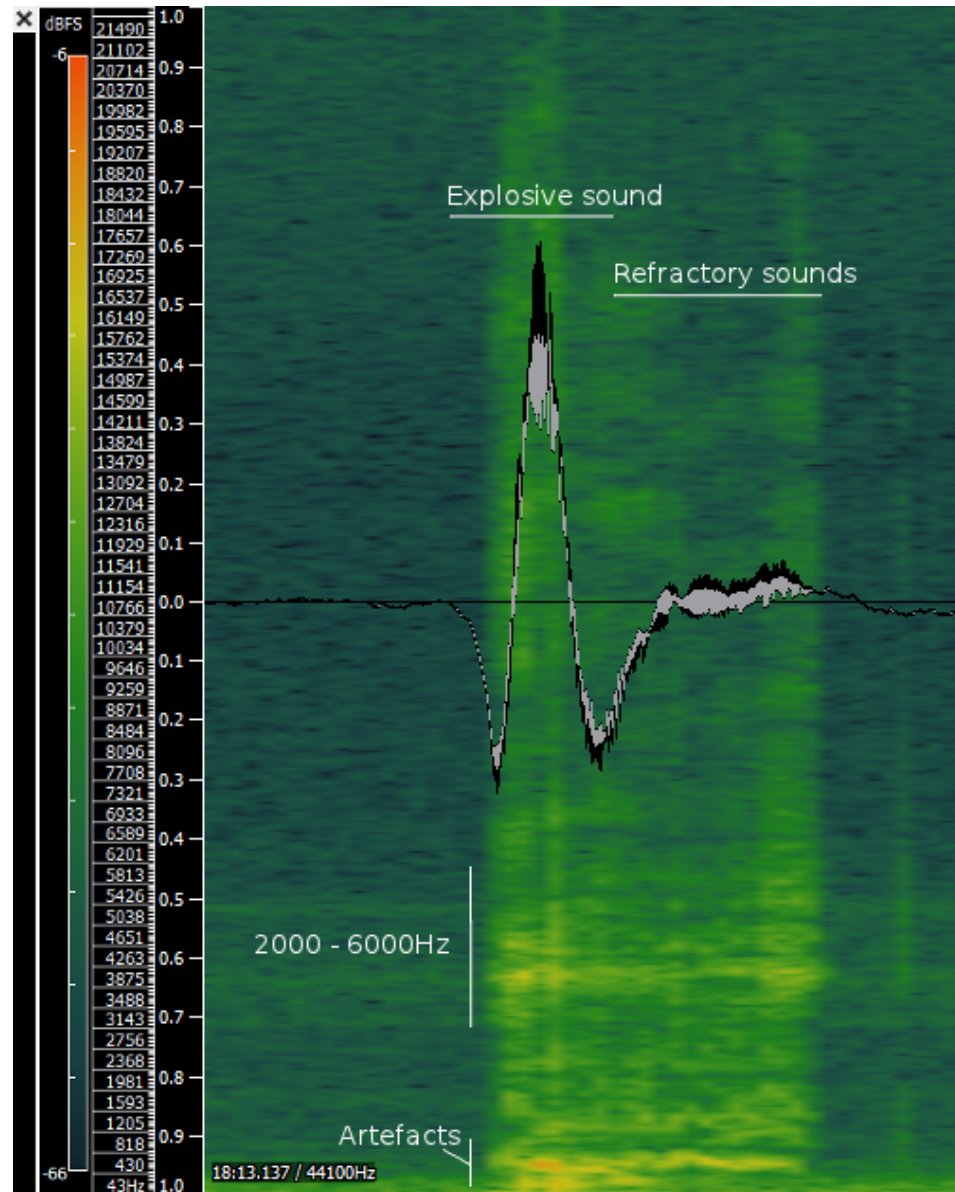


Figure 3.4: A prototypical cough in the rabbit. They each consist of a large singular explosive event followed by refractory sound. The explosive phase lasts between 80-500ms. The heat map underneath each audio signal represents the discrete Fourier transform of the audio signal binned at 1024 sample intervals. The region between 2000 and 6000Hz has been highlighted as a region of interest and the region marked artifact indicated noise related to the fume hood.

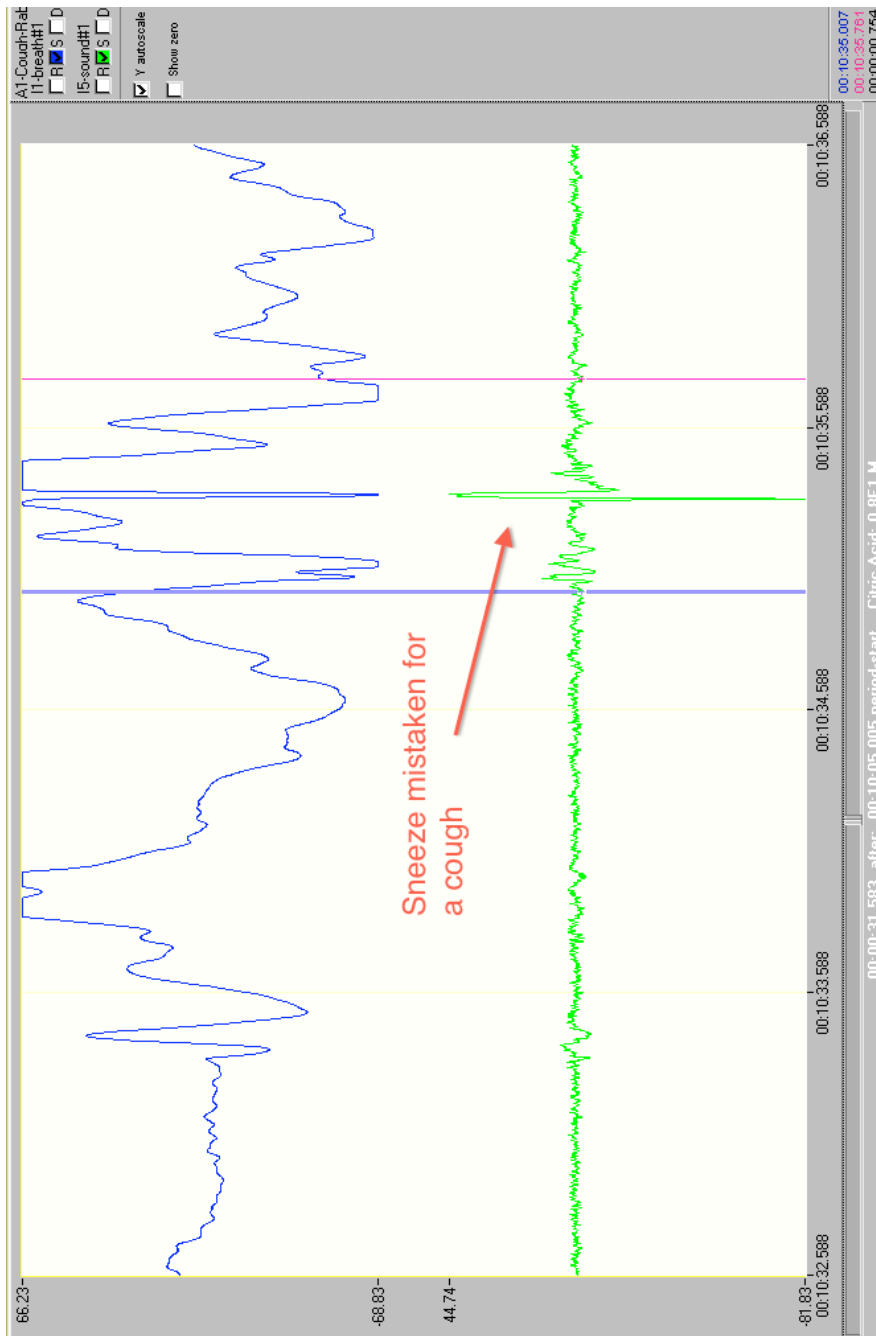


Figure 3.5: A sneeze that was mistakenly identified as a cough in the rabbit by the EMKA cough classifier. The threshold for the sound and pressure signal was triggered but manual classification made by listening to the signal revealed that the event was a sneeze.

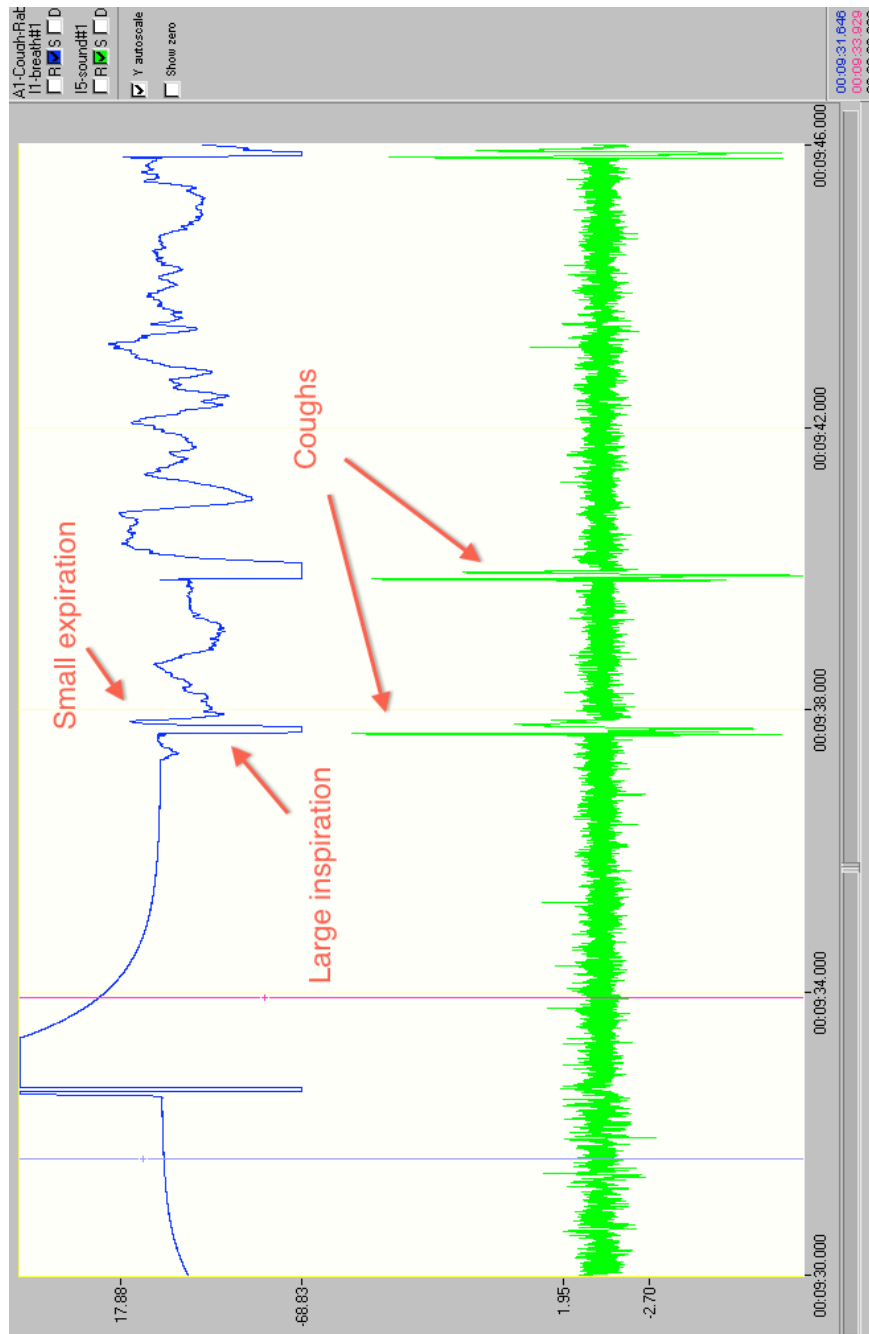


Figure 3.6: A cough with a large inspiration but small expiration that was mistakenly not identified as a cough in the rabbit by the EMKA cough classifier. The threshold for the sound but not the pressure signal was triggered. Manual classification made by listening to the signal revealed that the event was a cough.

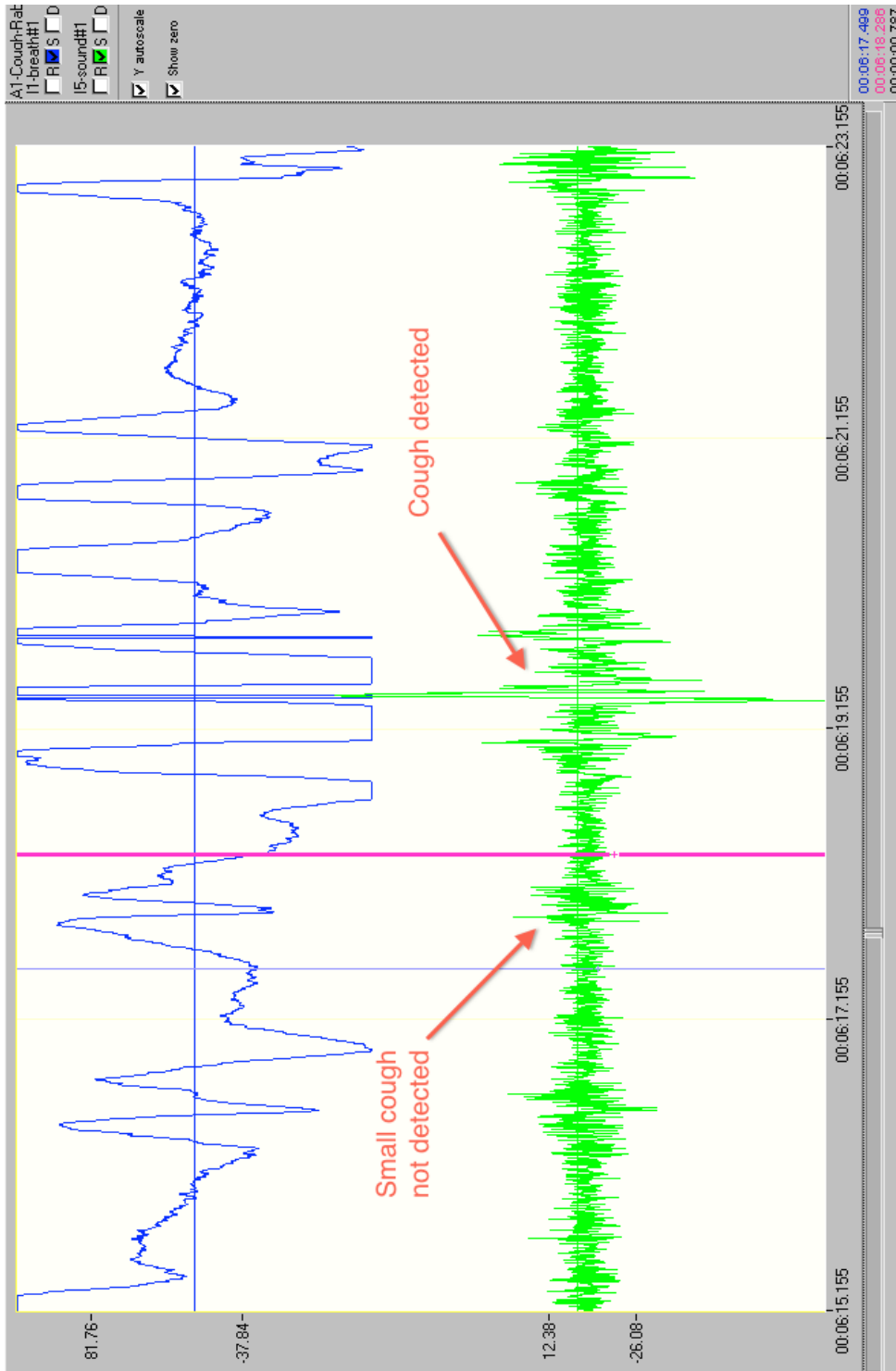


Figure 3.7: A screenshot of a quieter cough that was not classified by the EMKA cough classifier. The cough is quieter but the pressure signal suggests that a cough has occurred, listening to the audio signal confirmed that it was a cough.

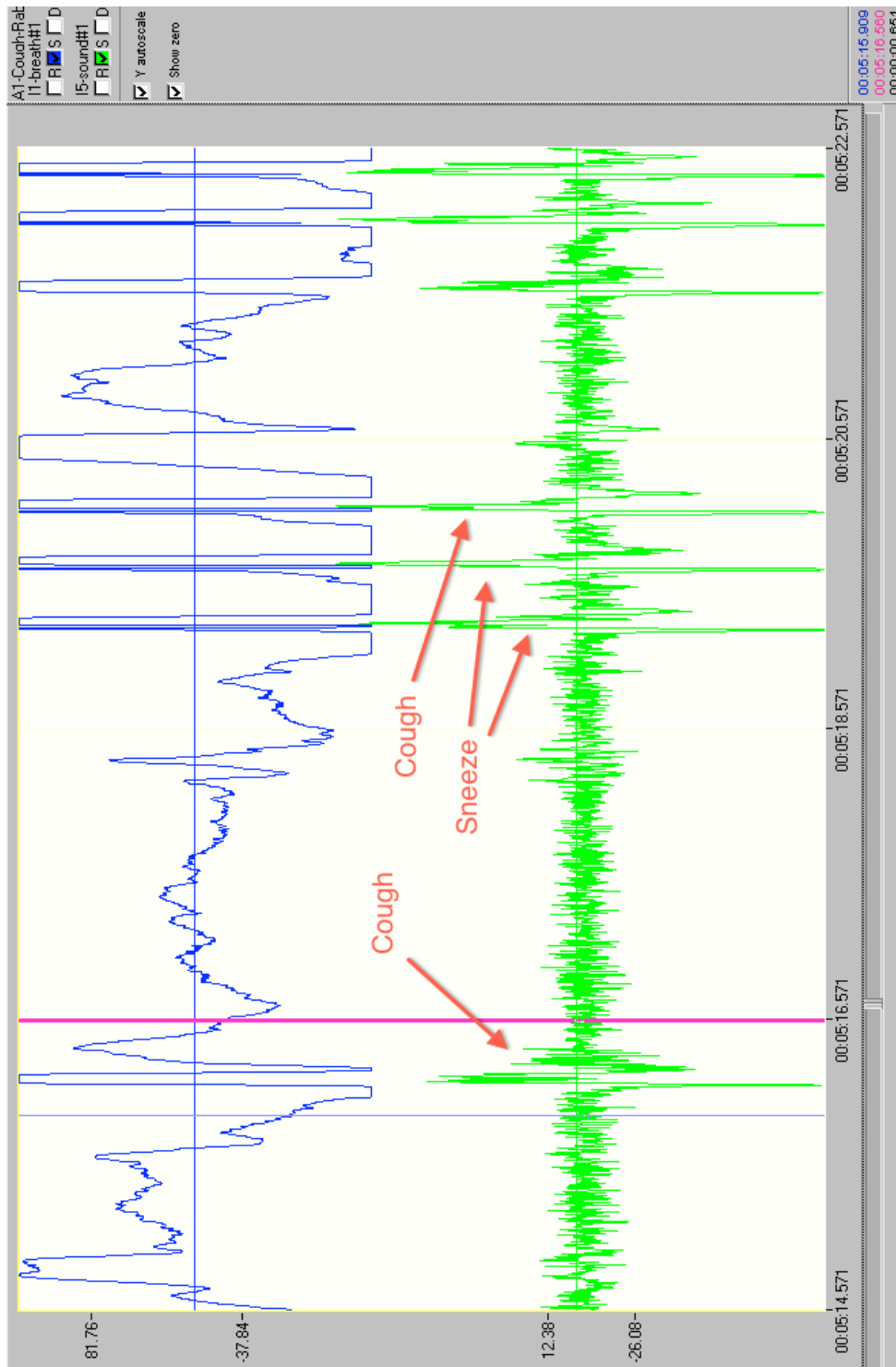


Figure 3.8: A screenshot of two coughs interleaved with sneezes in a rabbit. Visual inspection of the signals is not sufficient to discriminate between a cough and a sneeze.

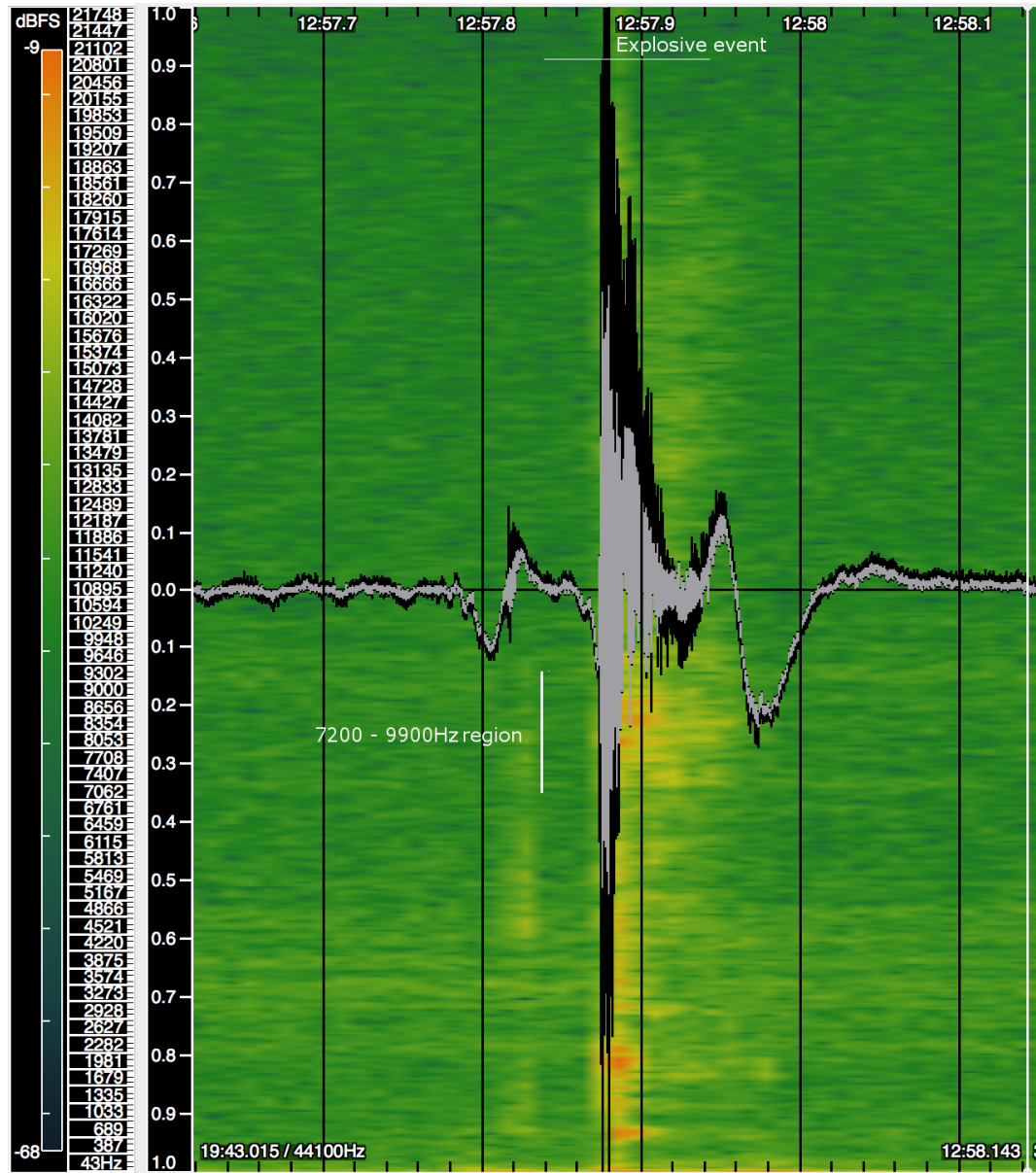


Figure 3.9: A prototypical cough event in a guinea pig. They each consist of a large singular explosive event, the explosive phase lasts around 300ms. The heat map underneath the audio signal represents the discrete Fourier transform of the audio signal binned at 1024 sample intervals. The frequency band common to most coughs has been highlighted at around 7200 to 9900Hz.

3.1.2 Citric acid induced cough

3.1.3 Rabbits

Rabbits may or may not respond to citric acid on their first occasion (see table 3.1) and, if they respond, the response invariably involves both coughs and sneezes.

Table 3.1: The total number of rabbits that coughed on their first naïve exposure to citric acid

Dose	Responders	Total Number of Rabbits	Response Rate
0.4M	3	8	38%
0.8M	33	40	83%
1.6M	4	4	100%

Rabbits respond well to citric acid at 1.6M, but the proportion responding to citric acid is greatly reduced at doses of 0.4M and 0.8M (Table 3.1). Of those rabbits that do respond, they respond well and while the response varies greatly the response approximates a concentration-dependant response (fig. 3.10). Repeated daily administration of citric acid, every 24 hours for 15 minutes, causes desensitization of the cough response in the rabbits (fig. 3.11).

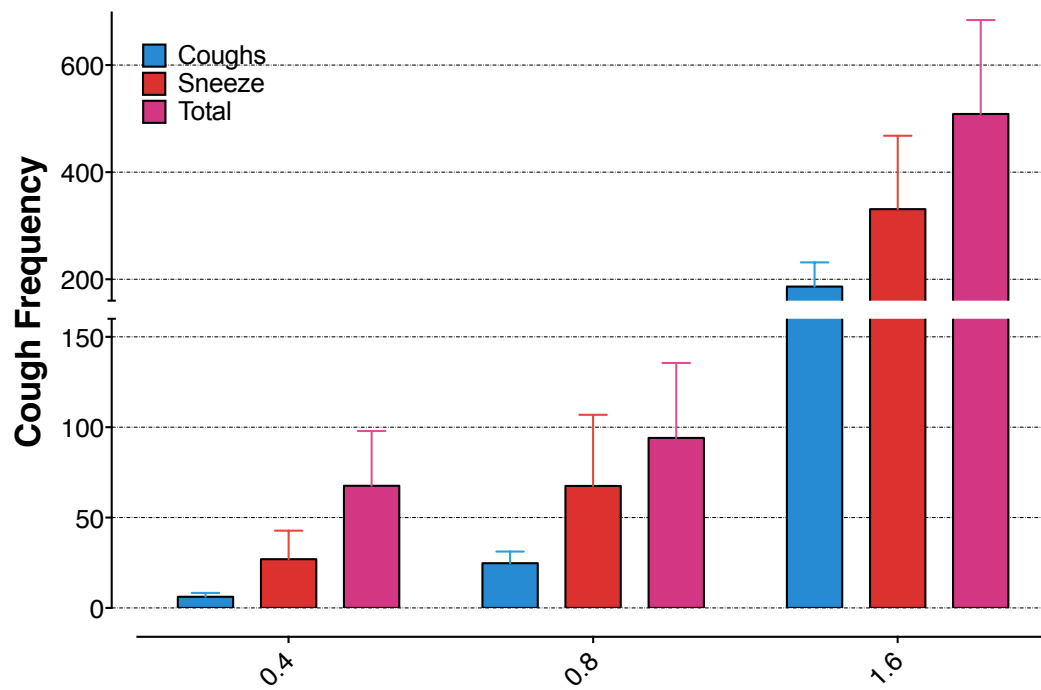


Figure 3.10: The cough, sneeze and total response for each rabbit that responded to citric acid on their screen. The error bars represent the SEM mean. 0.4M is n of 6, 0.8M is n of 40 and 1.6M is an n of 6.

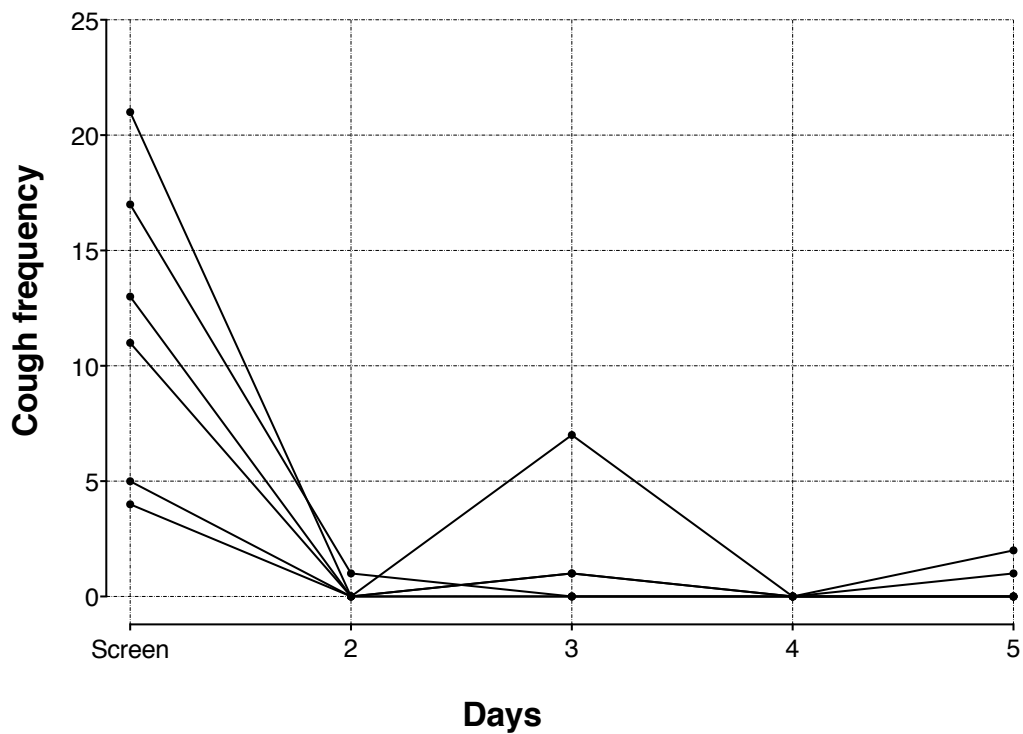


Figure 3.11: The cough response to citric acid over 5 consecutive days. Each rabbit was dosed with 0.8M each day for 5 days. n of 6.

3.1.4 Guinea-pigs

Guinea pigs respond well to citric acid and while the response varies greatly the response approximates a concentration-dependant response (fig. 3.10). Similar to rabbits, repeated daily administration of citric acid, every 24 hours for 15 minutes, causes desensitization of the cough response (fig. 3.13).

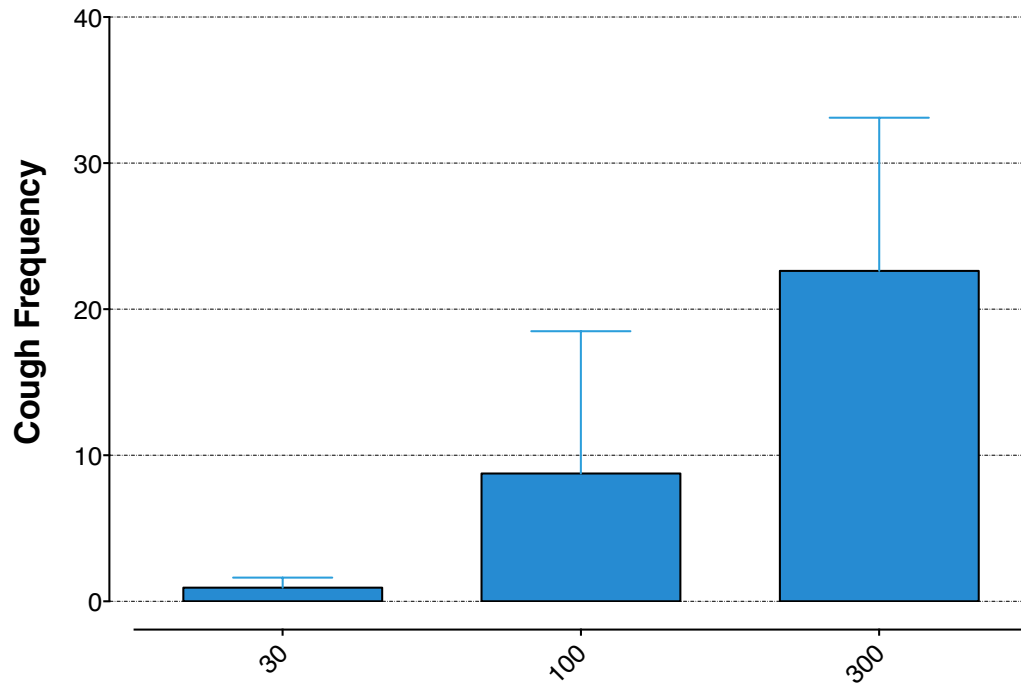


Figure 3.12: The cough for each guinea pig that responded to citric acid on their screen. The error bars represent the SEM. 30mM is n of 32, 100mM is n of 32 and 300mM is an n of 16.

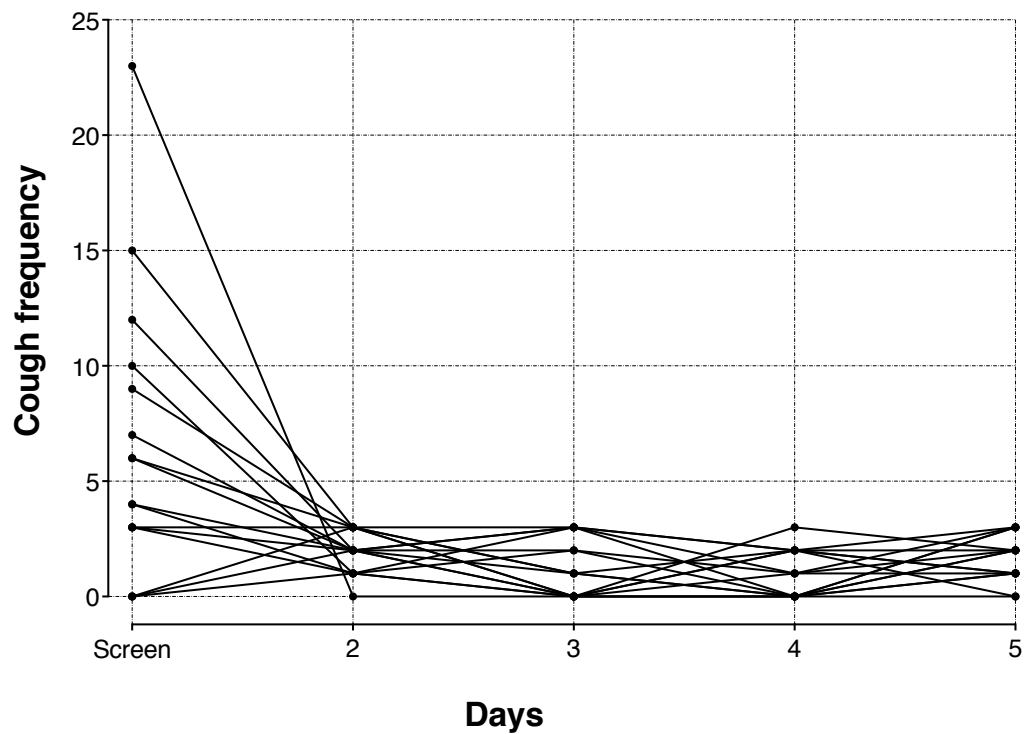


Figure 3.13: The cough response to citric acid over 5 consecutive days. Each guinea pig was dosed with 100mM each day for 5 days. n of 16.

3.2 Ozone and LPS sensitised hypertussive cough

3.2.1 The effect of ozone on citric acid induced cough

Ozone sensitization significantly increases cough response in both rabbits (fig. 3.14 and fig. 3.15) and guinea pigs (fig. 3.18 and fig. 3.19).

3.2.2 Rabbits

In rabbits, the cough frequency after treatment with ozone increased by 4.86 coughs (2.21 - 7.54, 95% CI) in the 400mM citric acid group and by 6.88 coughs (5.12 - 8.64, 95% CI) in the 800mM citric acid group. The results indicate that the cough response to citric acid is dose dependent ($p=0.010$ and $p=0.007$ respectively, t-test, two-tailed). Complimenting the increase in cough frequency was a responding and significant ($p < 0.0001$) decrease in the time-to-cough to citric acid, the time decreased from 58.1 seconds (52.7 - 63.5, 95% CI) in the control group to 22.9 seconds (21.4 - 24.4, 95% CI) in the ozone sensitised group (fig. 3.20A). Ozone can also sensitise the rabbits to cough without citric acid as a tussive stimuli - on one or more occasion, 74% of the rabbits studied (38 out of 52 rabbits) coughed an average of 16 coughs (7.15 - 20.51, 95% CI, data not shown) during the 1 hour ozone sensitization period.

Four rabbits in the high dose group were crossed over again and the response to air and ozone was reproducible (fig. 3.16). The difference between the crossed over ozone groups and the crossed over air groups was not significant.

Ozone exposure also shifted the ratio of coughs and sneezes and the relationship between coughs and sneezes became flatter (fig. 3.17).

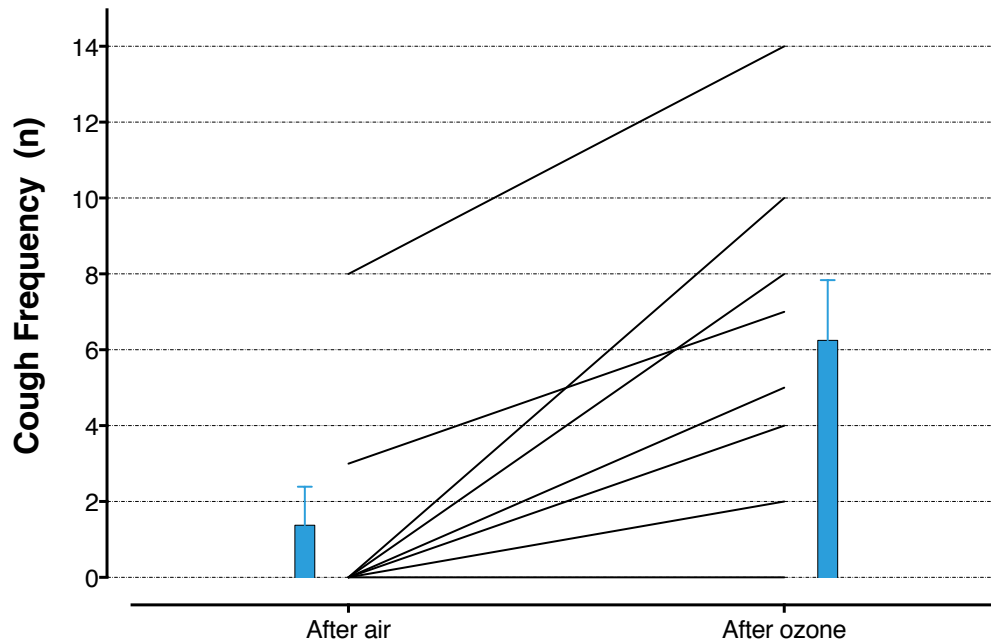


Figure 3.14: The frequency of coughs after the rabbit was given air paired with the frequency of coughs after the rabbit was given ozone at 2ppm. Ozone or air was given for 1hr immediately before being provoked into coughing using an aerosol of citric acid at 0.4M. The time period between each group, after air or after ozone was 7 days and the sequence of whether a rabbit received ozone or air first was randomised. n of 8. The blue bars indicate the mean response after air and after ozone and the error bars represent the SEM.

3.2.3 Guinea-pigs

Guinea pigs responded in a similar manner to the rabbits with the cough frequency after treatment with ozone increased by 9.25 (6.785 - 11.72, 95% CI) in the 30mM citric acid group and by 29.38 (14.33 - 44.42, 95% CI) in the 100mM citric acid group. Thus, similar to rabbits, the results indicate that the response is again dose dependent and was significant in both instances ($p < 0.008$ and $p < 0.0001$, respectively). Likewise, there was a significant ($p < 0.0001$) decrease in

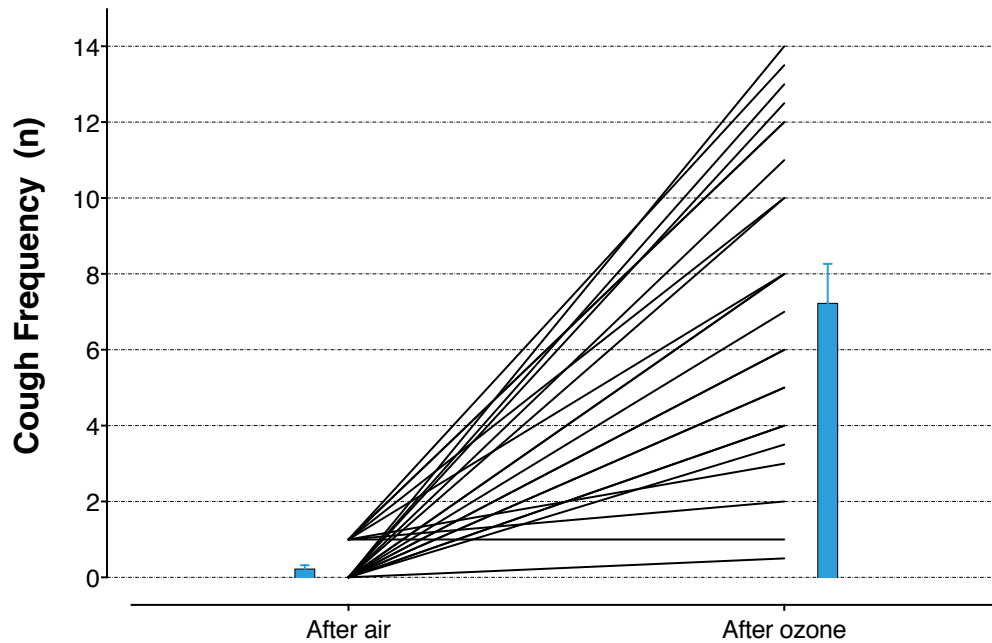


Figure 3.15: The frequency of coughs after the rabbit was given air paired with the frequency of coughs after the rabbit was given ozone at 2ppm. Ozone or air was given for 1hr immediately before being provoked into coughing using an aerosol of citric acid at 0.8M. The time period between each group, after air or after ozone was 7 days and the sequence of whether a rabbit received ozone or air first was randomised. n of 40. The blue bars indicate the mean response after air and after ozone and the error bars represent the SEM.

the time-to-cough to citric acid, the time decreased from 43.0 seconds (40.6 - 45.4, 95% CI) in the control group to 25.1 seconds (22.02 - 28.18, 95% CI) in the ozone sensitised group (fig. 3.20B).

In contrast, the confidence interval of the response is fairly consistent for the rabbit in the 400mM citric acid group and the 800mM group but in the guinea pig the 100mM citric acid group the confidence interval is much wider than the 30mM citric acid group. The difference between the time to cough in both

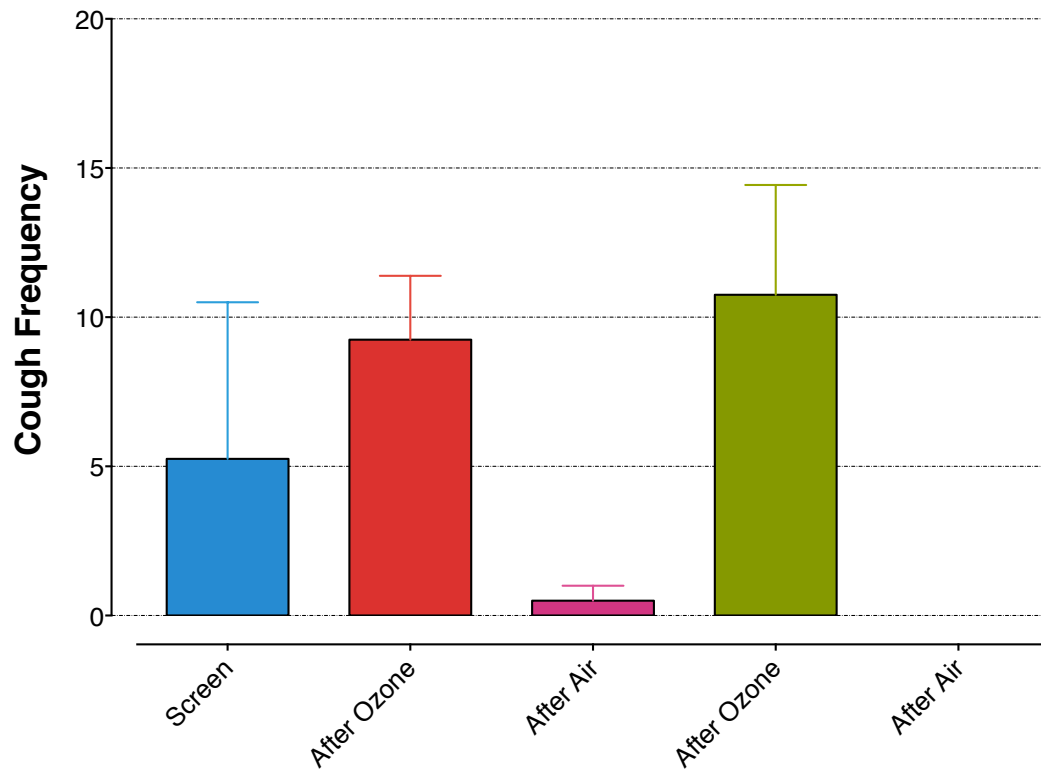


Figure 3.16: The effect of a repeat dose of ozone on the cough responses, the rabbits were dosed screened on day 0, exposed to either ozone or air on day 7, crossed over with the other treatment group on day 14, given the original treatment they received on day 7 on day 21 and then crossed over again on day 28. The error bar represent the SEM, n of 4

species was insignificant between the rabbit and guinea pig control groups (t-test, unpaired, $p > 0.31$) and the rabbit and guinea pig ozone groups (t-test, unpaired, $p > 0.27$).

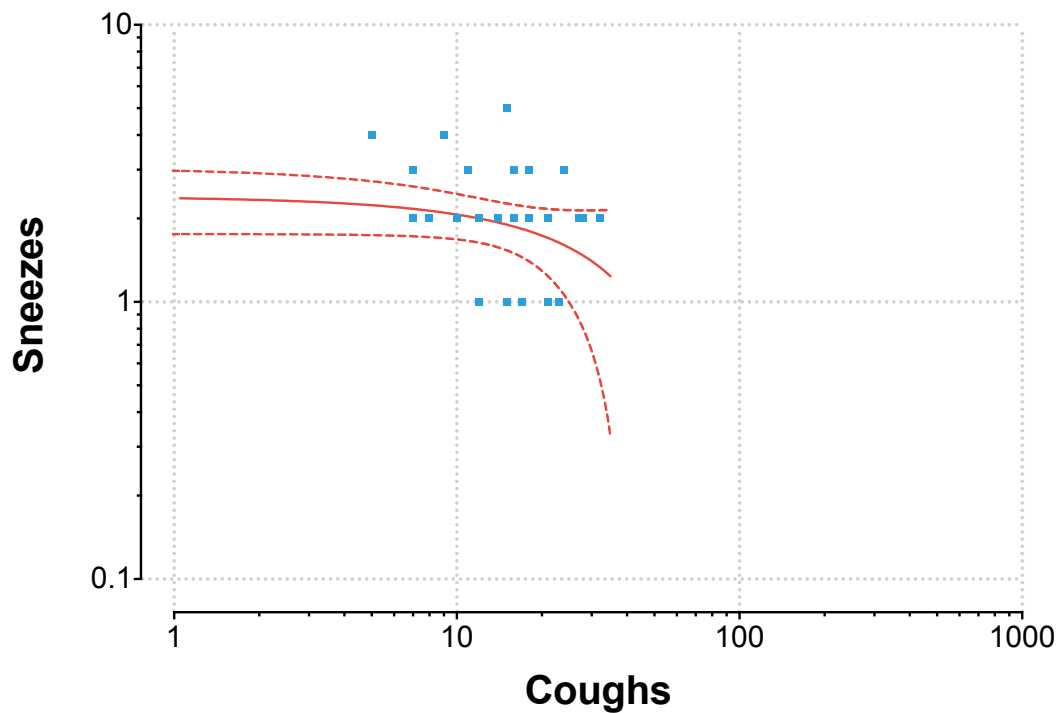


Figure 3.17: The correlation of coughs and sneezes for ozone-sensitized rabbits to citric acid, 0.8M. The solid line is an ordinary-least squares linear regression and the dashed line represents the 95% confidence intervals of that regression. n of 40.

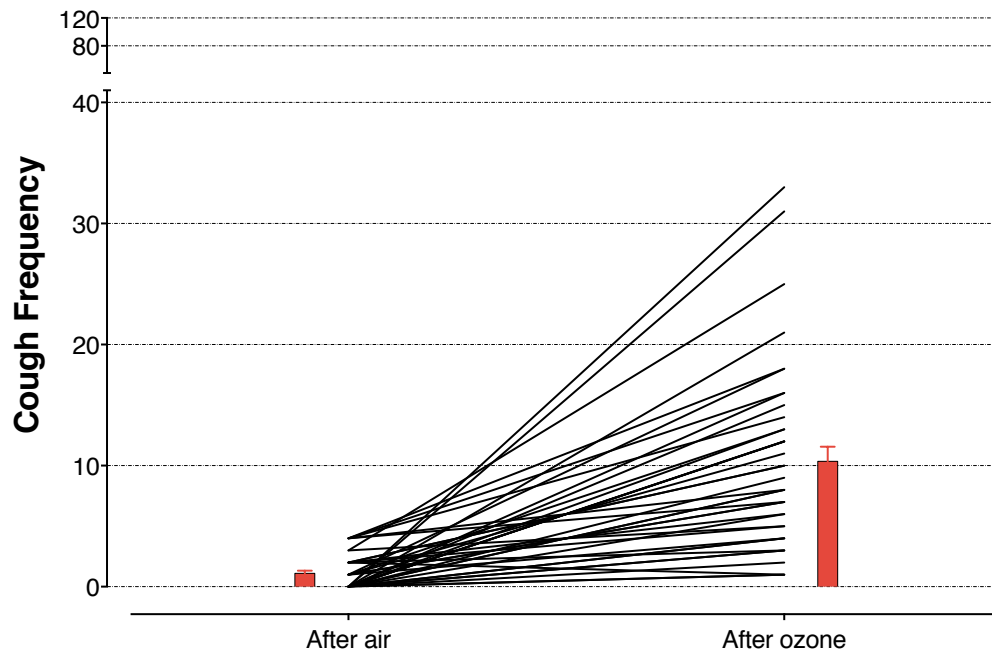


Figure 3.18: The frequency of coughs after the guinea pig was given air paired with the frequency of coughs after the guinea pig was given ozone at 2ppm. Ozone or air was given for 1hr immediately before being provoked into coughing using an aerosol of citric acid at 30mM. The time period between each group, after air or after ozone was 7 days and the sequence of whether a guinea pig received ozone or air first was randomised. n of 40. The blue bars indicate the mean response after air and after ozone and the error bars represent the SEM.

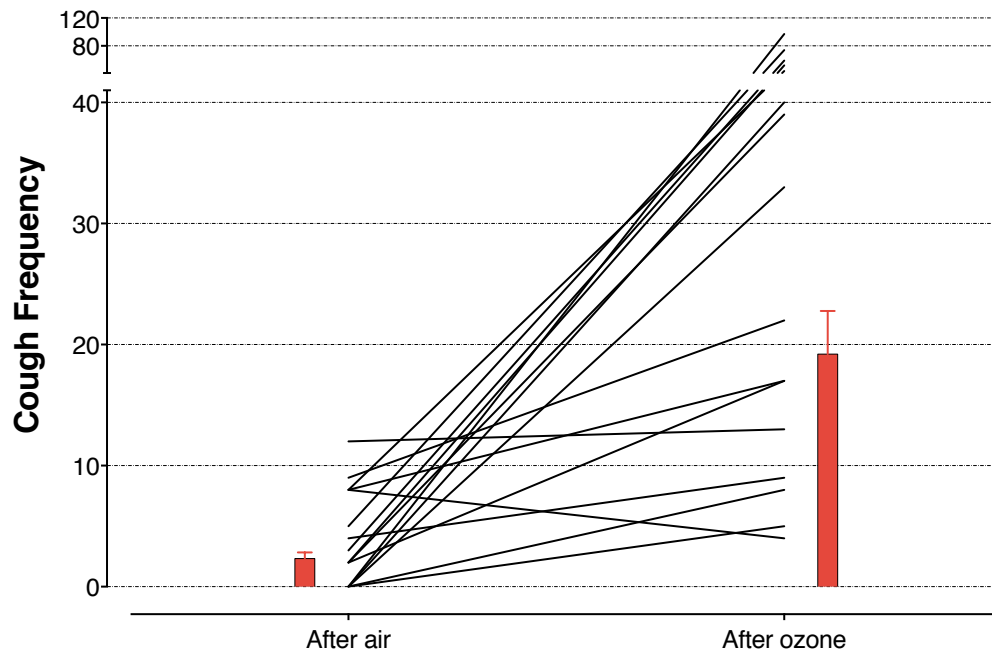


Figure 3.19: The frequency of coughs after the guinea pig was given air paired with the frequency of coughs after the guinea pig was given ozone at 2ppm. Ozone or air was given for 1hr immediately before being provoked into coughing using an aerosol of citric acid at 100mM. The time period between each group, after air or after ozone was 7 days and the sequence of whether a guinea pig received ozone or air first was randomised. n of 16. The blue bars indicate the mean response after air and after ozone and the error bars represent the SEM.

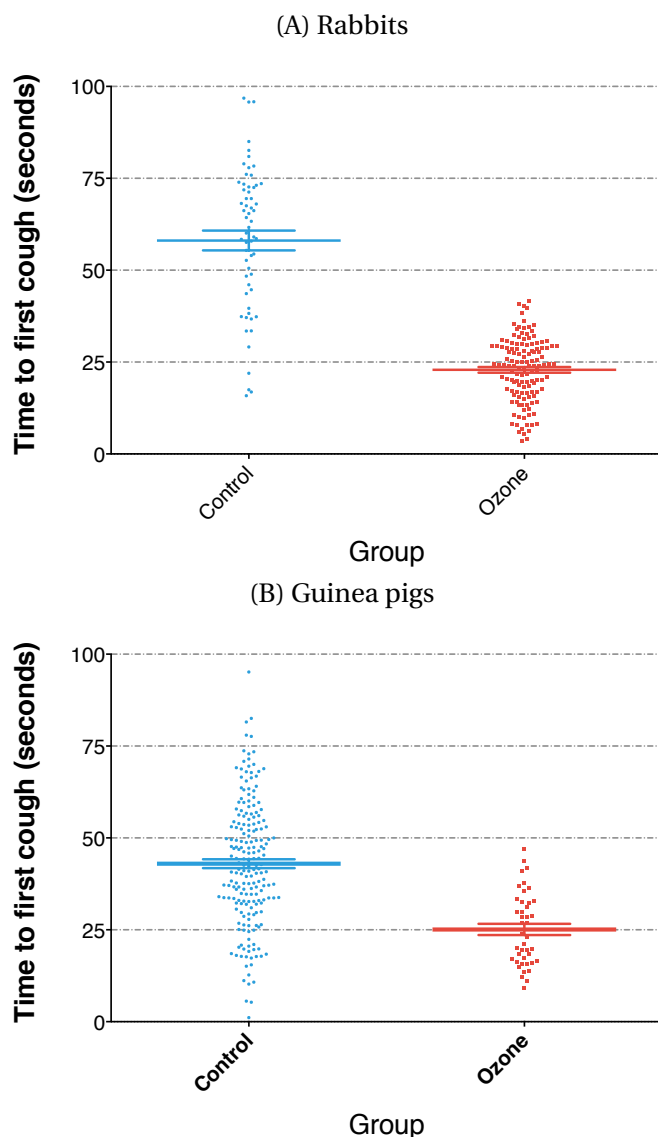


Figure 3.20: The effect of ozone on the time-to-first-cough in both rabbits (3.20A) and guinea pigs (3.20B) which is the time in seconds when the first cough occurred after the nebuliser that vapourised citric acid was turned on. The thick horizontal bar represents the mean and the error bars are the SEM, n of 48 for the rabbit control group and 129 for ozone group, n of 198 for the guinea pig controls and 48 for the ozone sensitised guinea pigs. Please note that animals had a varying number of repeats of ozone sensitisation but were only screened once, n numbers is a aggregation of data from multiple protocols.

3.2.4 The effect of LPS on citric acid induced cough in the guinea pigs

Guinea pigs given an intratracheal dose of LPS, 1ml of $50\mu\text{g ml}^{-1}$ 4 hours prior to exposure with citric acid failed to sensitise guinea pigs into a hypertussive cough in response to three doses of citric acid (fig. 3.21). The aerosol experiments are not shown as these were done as a pilot experiment before switching to intratracheal dosing only. Cell counting facilities were not available at the time of the experiments so there are no confirming neutrophil counts.

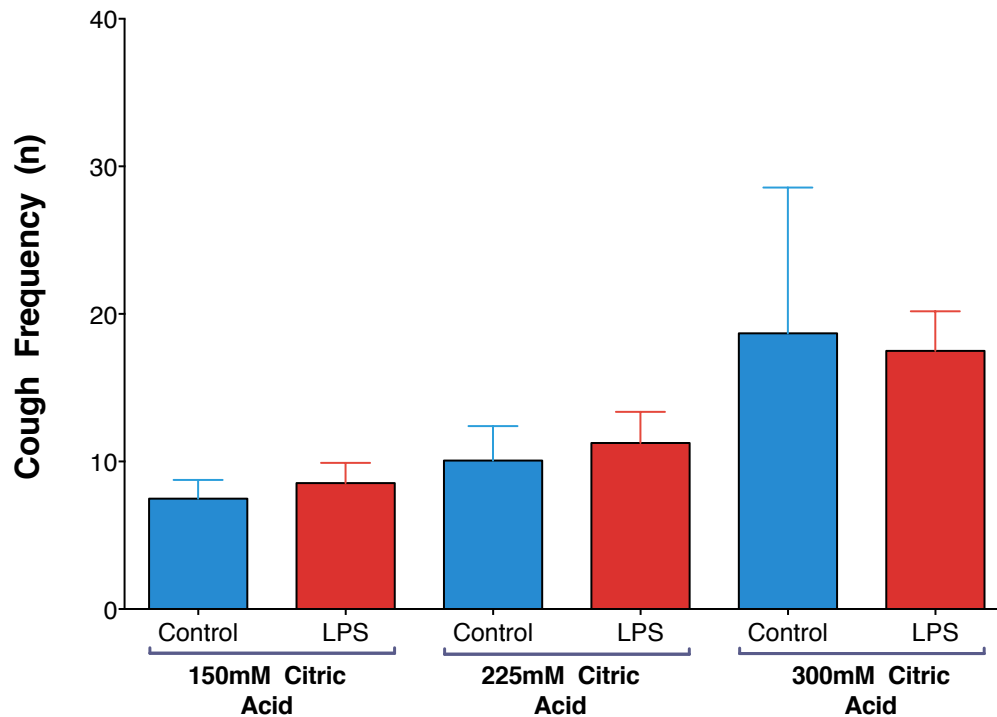


Figure 3.21: The effect of LPS on the citric acid induced cough in guinea pigs. The bars represent the mean and the error bars are the SEM, n of 16 each, group.

3.2.5 The effect of ozone on lung function

There was no significant difference between the baseline responses for C_{dyn} and R_L (using Sidak's multiple comparison) between the control and tiotropium bromide groups whether the rabbit had received ozone sensitization or not (fig. 3.22).

Methacholine caused a concentration dependent fall in C_{dyn} post-saline challenge and a rise in R_L post-saline challenge in both guinea pigs and rabbits (fig. 3.23).

In rabbits, the C_{dyn} PC_{35} to methacholine (mean \pm CI 95%) in the control group without ozone sensitization was 3.71 (2.65 - 3.18, 95% CI) mg ml^{-1} (fig. 3.23A) and was significantly lower in the control group with ozone sensitization at 1.34 (0.91 - 1.77, 95% CI) mg ml^{-1} methacholine (paired t-test, $p < 0.02$). Similarly, the R_L PC_{50} for the control group without ozone sensitization was 2.72 (2.08 - 3.34, 95% CI) mg ml^{-1} methacholine but was not significantly lower in the control group with ozone sensitization at 1.32 (0.80 - 1.84, 95% CI) mg ml^{-1} methacholine (paired t-test, $p = 0.22$). In effect, ozone shifted the C_{dyn} PC_{35} dose response curve to methacholine left by 0.33 log units and the PC_{50} R_L left by 0.21 log units.

The guinea pig was much more sensitive to methacholine than the rabbit, although the effect of ozone sensitization was very similar. The C_{dyn} PC_{35} to methacholine (mean \pm CI 95%) in the control group without ozone sensitization was 0.13 (0.067 - 0.19, 95% CI) mg ml^{-1} (fig. 3.23A) and was significantly lower in the control group with ozone sensitization at 0.09 (0.064 - 0.11, 95% CI) mg ml^{-1} methacholine (t-test, paired, $p < 0.05$). Similarly, the R_L PC_{50} for the control

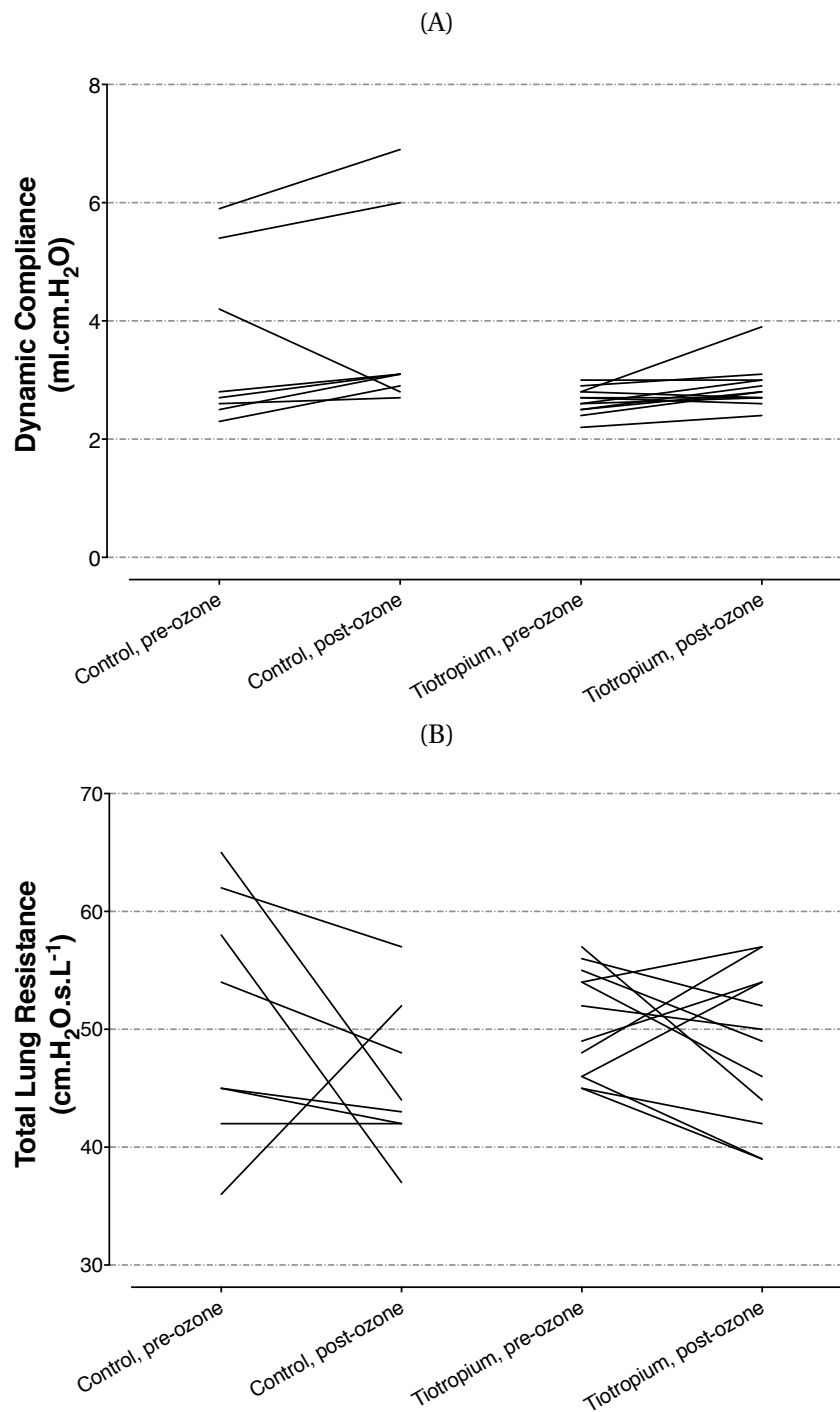


Figure 3.22: The baseline compliance (A) and resistance (B) for the control group pairwise with and without ozone treatment in rabbits. Control group n of 8 and tiotropium bromide group n of 12. 130

group without ozone sensitization was 0.23 (0.15 - 0.31, 95% CI) mg ml^{-1} methacholine and was significantly lower for the post-ozone control group (t-test, $p < 0.05$) at 0.10 (0.058 - 0.14, 95% CI) mg ml^{-1} methacholine. Ozone shifted the C_{dyn} PC_{35} dose response curve to methacholine left by 0.16 log units and the PC_{50} R_L left by 0.36 log units.

Guinea pigs (n of 8) were dosed acutely, immediately after ozone sensitization the lung function experiment was performed. In these guinea pigs, the response to methacholine was the same as the pre-ozone control group indicating that the effect of ozone on lung function involves a latent response to the effect of ozone on the airways.

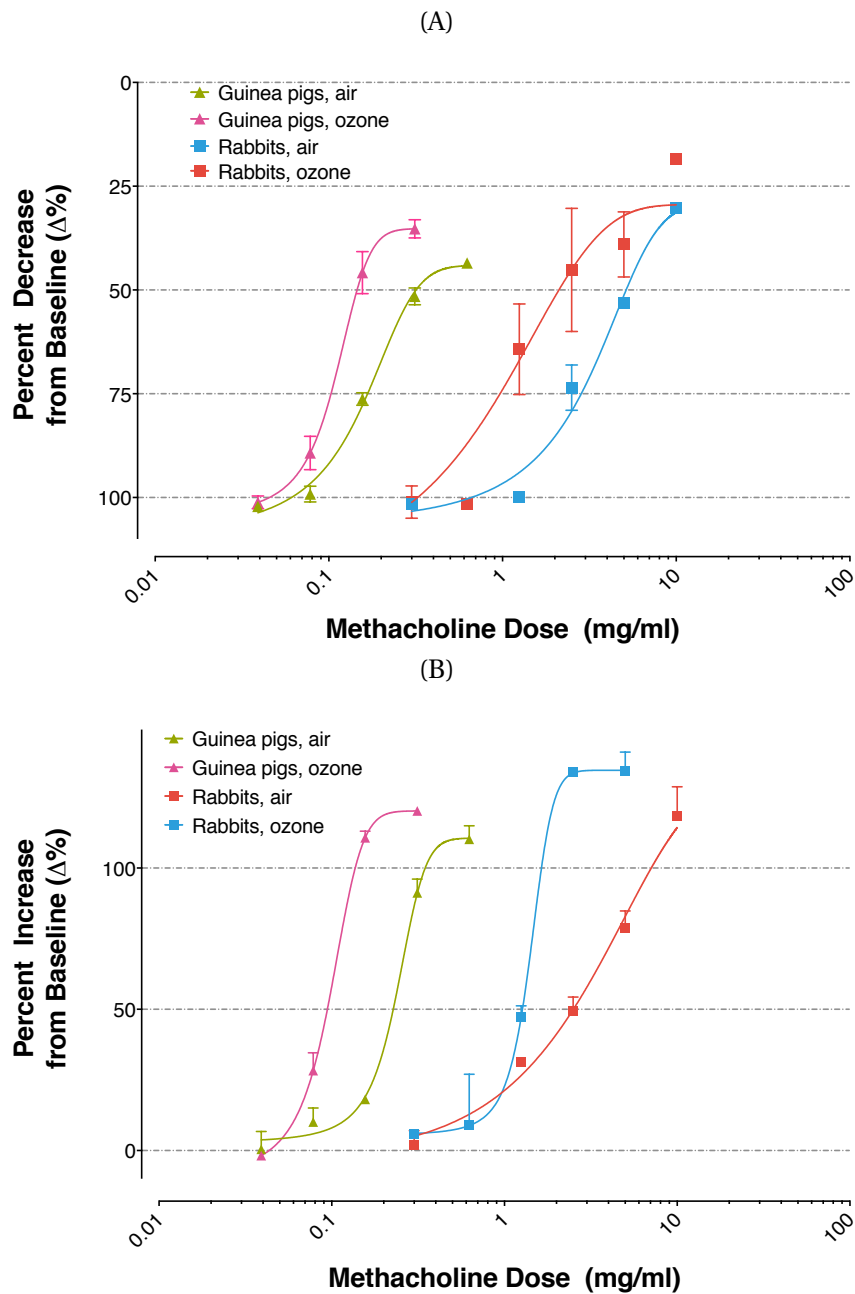


Figure 3.23: The dose response curve of dynamic compliance (A) and total lung resistance (B) to methacholine with and without ozone sensitization in rabbits (■) and guinea pigs (▲). n of 8 for rabbits paired, n of 8 for guinea pigs in each group.

3.2.6 The effect of ozone on pulmonary leukocyte recruitment

In rabbits, 4 hours after a 1hr exposure to 2ppm ozone the total cell count in the control group increased fourfold from 0.57 (0.46 - 0.74, 95% CI) $\times 10^6$ cells/ml to 2.19 (0.50 - 3.88, 95% CI) $\times 10^6$ cells/ml (fig. 3.24) and this difference was significant (t-test, paired, $p < 0.001$). The differential cell count indicated a significant (paired t-test, $p < 0.001$) increase in neutrophils after ozone challenge, from 0.71 $\times 10^4$ /ml (0.30 - 1.11 $\times 10^4$, 95% CI) to 5.41 $\times 10^5$ /ml (3.02 - 7.80 $\times 10^5$ /ml, 95% CI) and a significant increase in monocytes from 5.27 $\times 10^5$ /ml (3.93 - 6.62 $\times 10^5$ /ml, 95% CI) pre-ozone to 1.64 $\times 10^6$ (0.28 - 3.23 $\times 10^6$, 95% CI) post-ozone (paired t-test, $p < 0.001$). There were no remarkable changes in eosinophils.

In guinea pigs, 4 hours after a 30 minute exposure to ozone (2ppm) the total cell count in the control group increased sixfold from 0.39 $\times 10^5$ cells/ml (0.28 - 0.49 $\times 10^5$ cells/ml, 95% CI) to 2.32 $\times 10^5$ cells/ml (1.69 - 2.95 $\times 10^5$ cells/ml, 95% CI) (fig. 3.25) and this difference was significant (paired t-test, $p < 0.0001$). The differential cell count indicated a significant increase in neutrophils after ozone challenge, from 5.61 $\times 10^2$ /ml (1.64 - 12.8 $\times 10^2$ /ml, 95% CI) to 1.2 $\times 10^5$ cells/ml (0.77 - 1.68 $\times 10^5$ cells/ml, 95% CI) and a decrease in monocytes from 3.8 $\times 10^4$ /ml (2.75 - 4.89 $\times 10^4$ /ml, 95% CI) pre-ozone to 1.09 $\times 10^5$ cells/ml (0.60 - 1.58 $\times 10^5$ cells/ml, 95% CI) post-ozone. Similarly to rabbits, there were no significant changes in eosinophils.

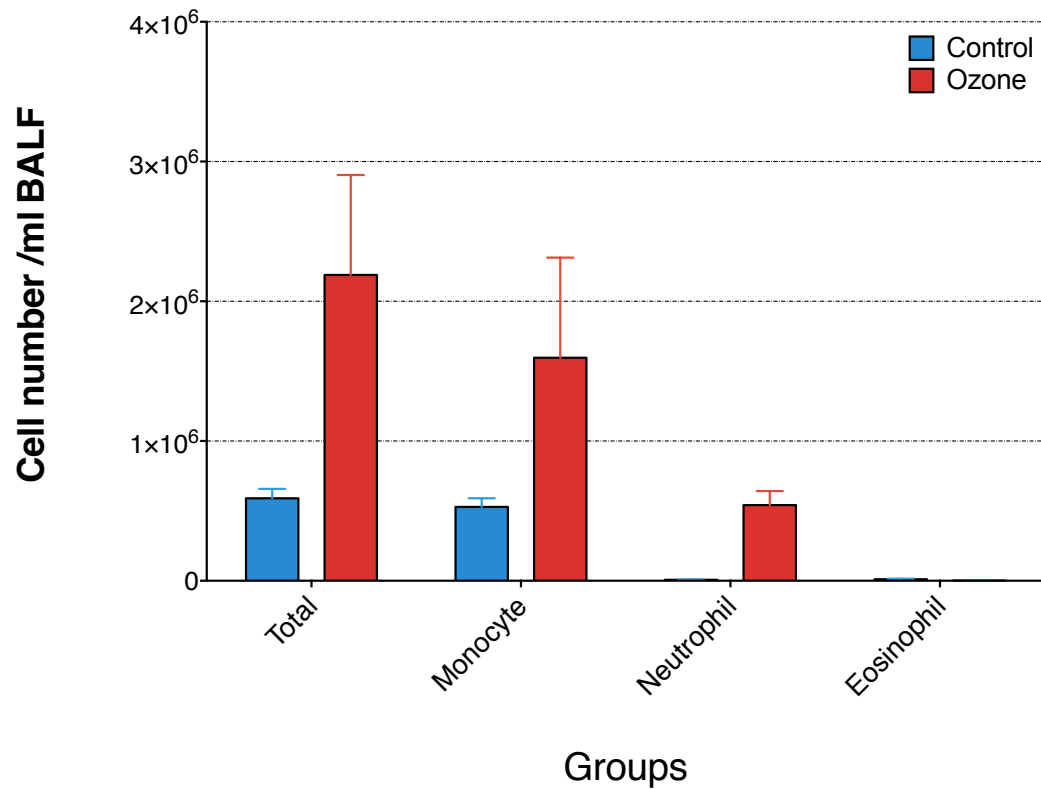


Figure 3.24: The change in the total and differential cell count before ozone and after ozone sensitization in the control group. On day one, rabbits were exposed to air for 1 hour and lavaged 4 hours later, this was the control group. On day three, the same rabbits, were exposed to ozone (2ppm) for 1 hour and lavaged 4 hours later, this was the ozone group. The bars represent the mean and the error bars are the SEM, n of 8.

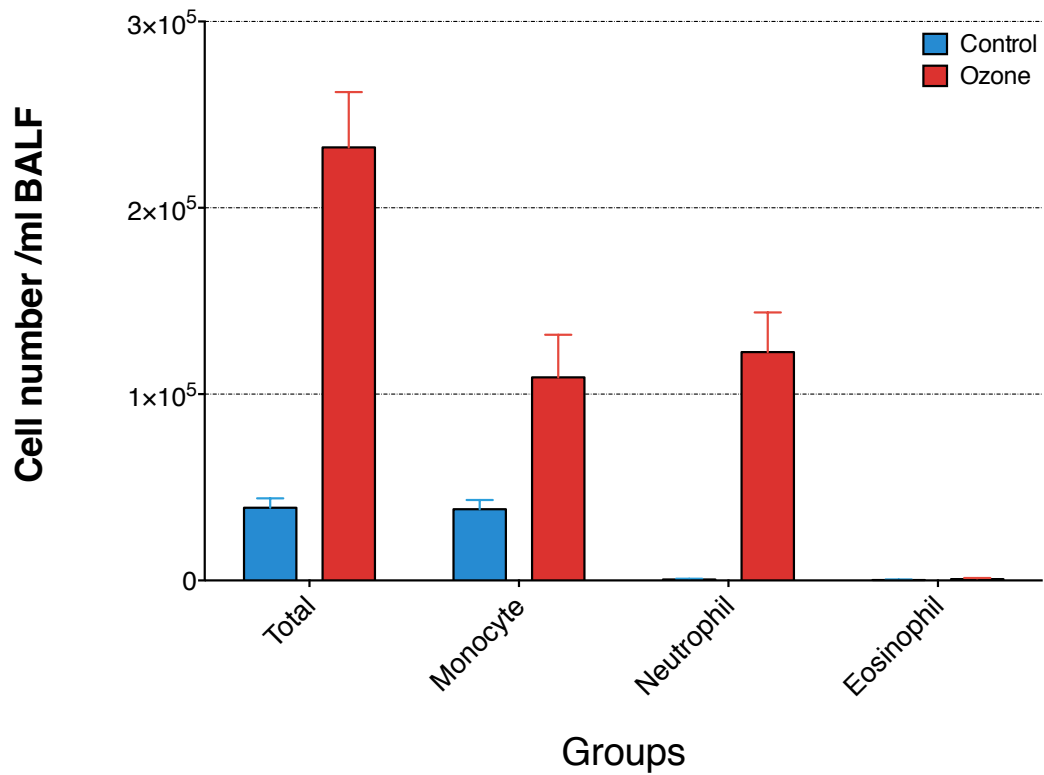


Figure 3.25: The change in total cell and differential count for before ozone and after ozone in the control group. Guinea pigs in the control group were exposed to air for 1 hour while guinea pigs in the ozone group were exposed to ozone (2ppm) for 1 hour. 4 hours later, they were tracheotomized and immediately lavaged. The bars represent the mean and the error bars are the SEM, n of 16, each group.

3.2.7 The mechanism of action of ozone sensitisation

Chronic capsaicin pretreatment in the rabbit abolished the ozone-sensitised response to citric acid (paired t-test, $p < 0.05$), implying that ozone is sensitising the rabbit to cough via peripheral sensory nerves (fig. 3.26). TRPA1 was considered a candidate target for ozone sensitization and cinnamaldehyde was used as prototypical TRPA1 agonist. Cinnamaldehyde, at a very high concentration of 30M, in an ozone sensitised rabbit failed to elicit a significant cough response in the rabbits when compared to citric acid in an ozone sensitised rabbit (One-way ANOVA, Tukey comparison post-hoc, Ozone + Citric Acid vs Ozone + Cinnamaldehyde 30M) (fig. 3.27), however, cinnamaldehyde at 800mM elicited a significant cough response in guinea pigs (One-way ANOVA, Tukey comparison post-hoc, Cinnamaldehyde 800mM vs Screen) confirming the results of Birrell *et al.* (2009) who also used 800mM cinnamaldehyde in their experiments. The lower dose of 30mM, specified by Brozmanova *et al.* (2012), did not elicit a cough response (One-way ANOVA, Tukey comparison post-hoc, Cinnamaldehyde 30mM vs Screen). HC-030031, a TRPA1 antagonist significantly attenuated the cinnamaldehyde induced cough response (paired t-test, $p < 0.01$) but failed to attenuate the ozone sensitised citric acid induced cough (section 3.2.7).

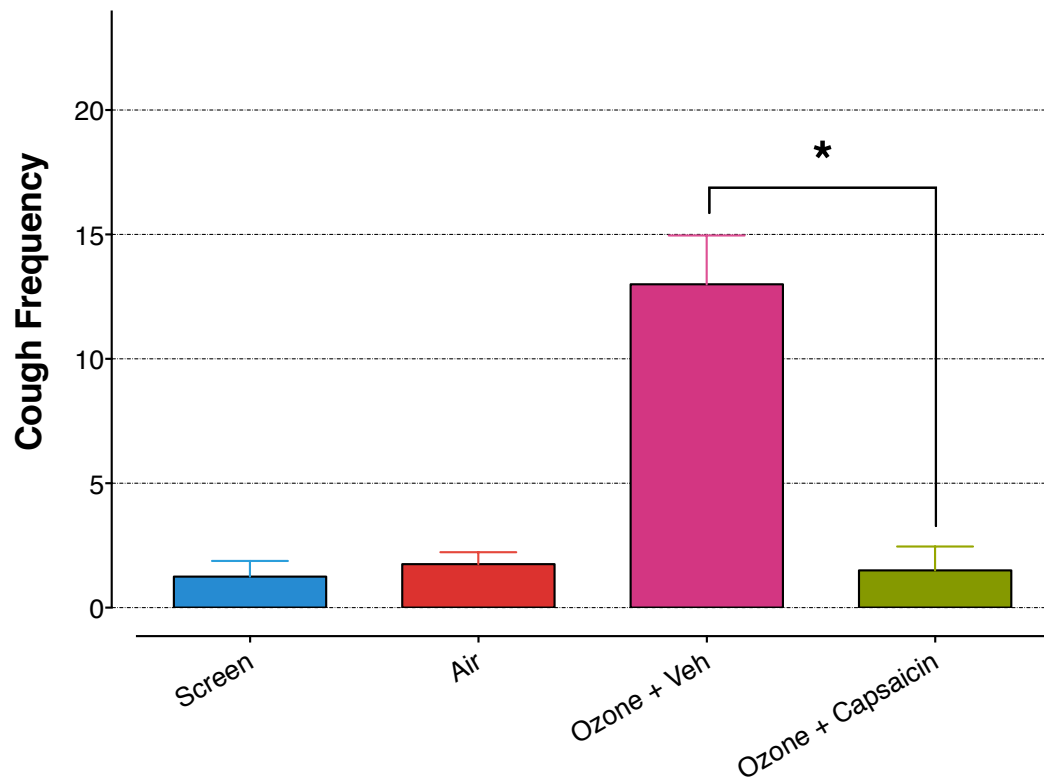


Figure 3.26: The effect of capsaicin desensitization on the cough response in rabbits, the rabbits were treated with capsaicin on day 1 to 3 (see section 2.9.3) and on day 6 were sensitised with ozone and provoked into coughing using citric acid, 0.8M. The error bar represents the mean \pm SEM, n of 4. “*” represents $p < 0.05$, paired t-test.

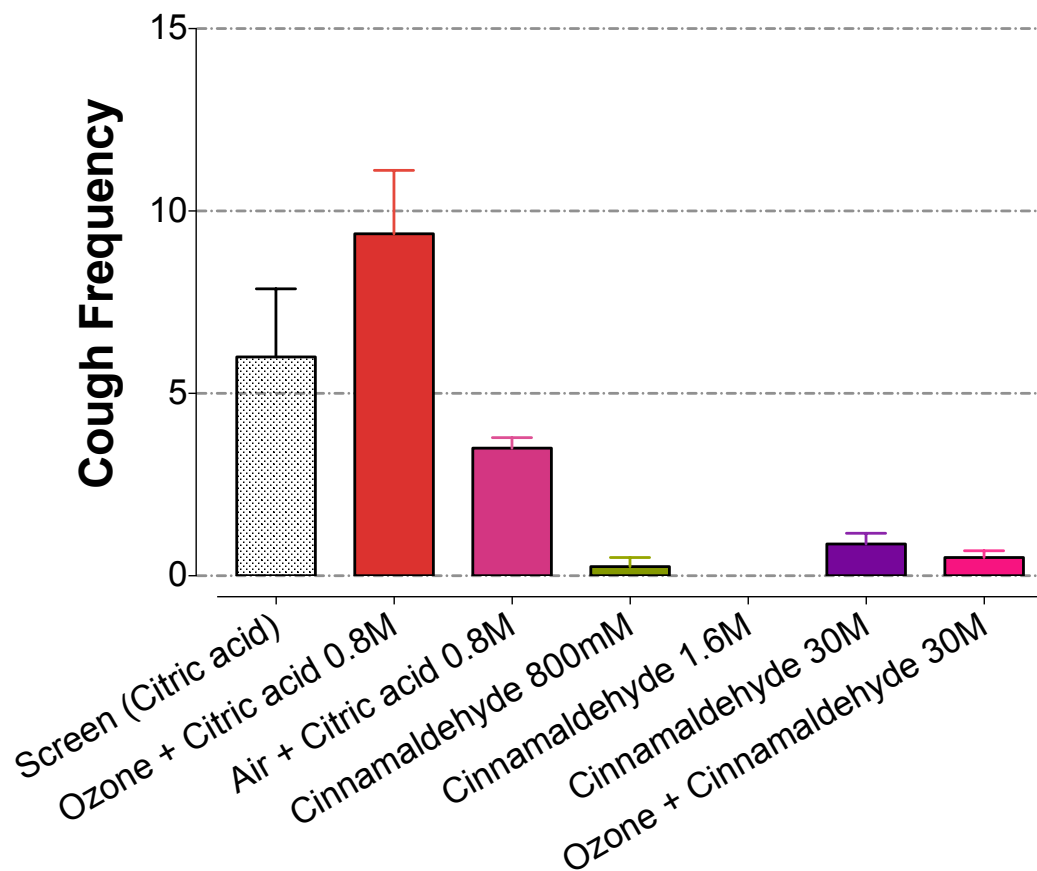


Figure 3.27: The cinnamaldehyde induced cough response in rabbits with and without ozone sensitization and with and without HC-030031 treatment. Each animal had each treatment at 7 day intervals and received the treatments in a randomised order. n of 4, the error error bars represent the SEM.

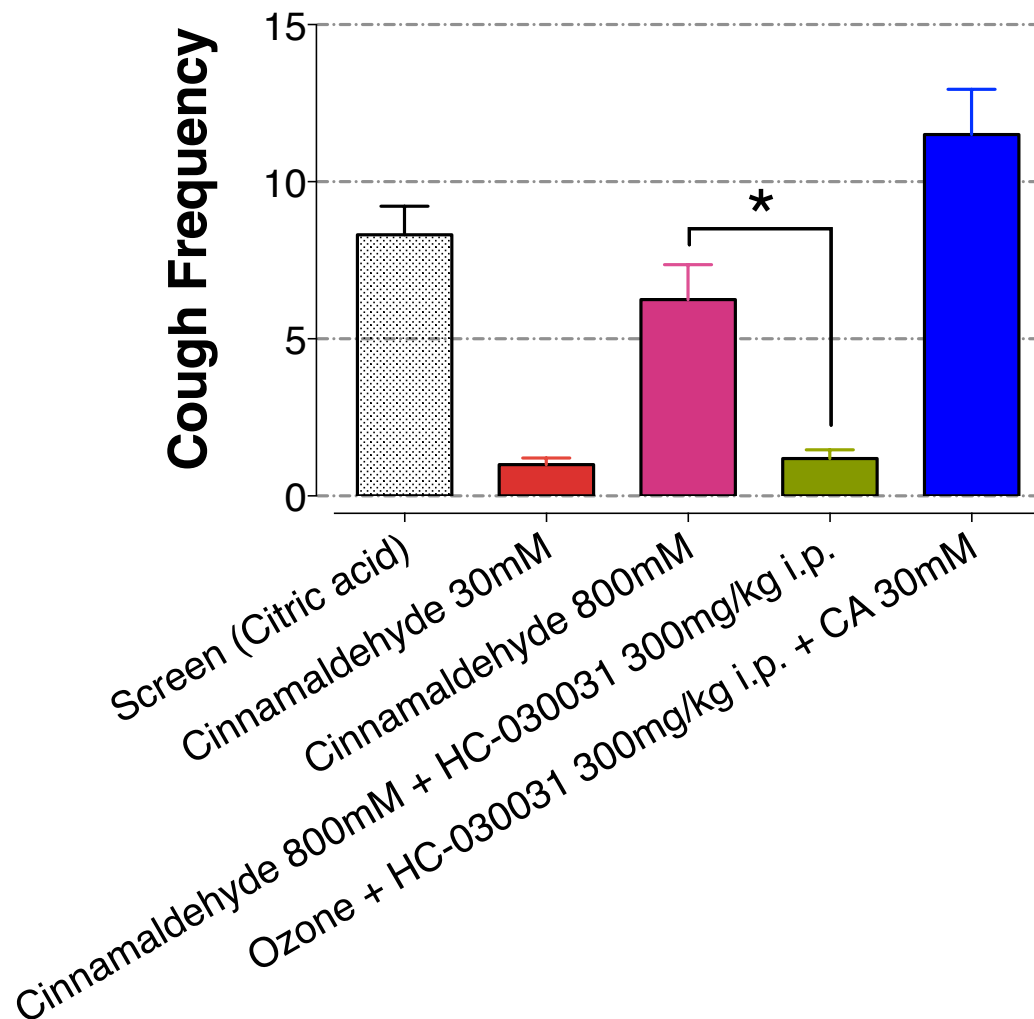


Figure 3.28: The cinnamaldehyde induced cough response in guinea pigs with and without ozone sensitization and with and without HC-030031 treatment. Each animal had each treatment at 7 day intervals and received the treatments in a randomised order. n of 4, the error bars represent the SEM. “*” represents $p < 0.05$.

3.3 Validating current antitussives and evaluating putative antitussives

3.3.1 Rabbits

There were significant differences between the treatment groups in the rabbit (repeat measures ANOVA) given at 7 day intervals but the repeat measures were effectively matched ($p < 0.0001$) and sphericity was not violated ($p < 0.05$).

In rabbits, treatment with roflumilast (1 mg kg^{-1} , i.p.) and salbutamol ($100 \mu\text{g ml}^{-1}$, aerosol, 2 mins at 3 L/min) did not significantly alter the ozone-sensitised response to citric acid induced cough in rabbits (post-hoc Dunnett's, fig. 3.29). However, treatment with codeine significantly reduced (post-hoc Dunnett's) the ozone-sensitised response by a factor of 2 from 14.8 (10.2 - 19.4, 95% CI) to 6.25 (0.97 - 11.53, 95%).

3.3.2 Guinea pigs

The sequence and period of the treatments did not have a significant effect on the cough response (Mixed Linear Model, $\text{Pr}>F > 0.05$ and $\text{Pr}>F > 0.05$, respectively). There were significant differences between the treatment groups given at 7 day intervals (repeat measures ANOVA and Mixed Linear Model, $\text{Pr}>F < 0.01$) and the groups were effectively matched ($p < 0.001$) and sphericity was not violated ($p < 0.01$).

In guinea pigs, treatment with roflumilast (1 mg kg^{-1} , i.p.) did not significantly alter the ozone-sensitised response to citric acid (post-hoc

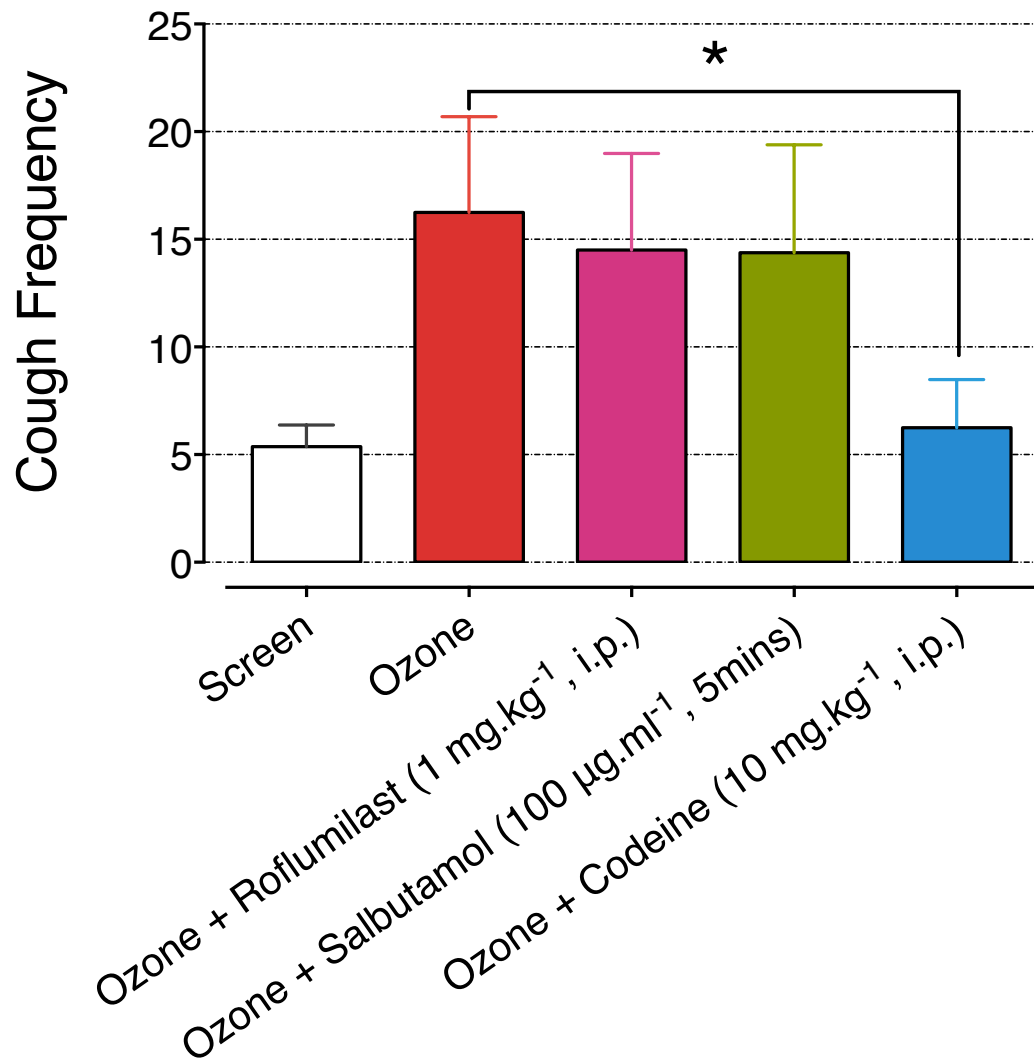


Figure 3.29: The effect of various putative and known antitussives on the cough response in rabbits. The error bar represent the mean \pm SEM, n of 8. The connecting lines between bars represents statistical significance, “*” represents $p < 0.5$.

Dunnett's). However in contrast to rabbits, salbutamol ($50 \mu\text{g ml}^{-1}$, aerosol, 2 mins at 3 L/min) significantly altered the ozone-sensitised response to citric acid induced coughing (t-test, two-tailed, $p < 0.001$) from 16.6 coughs (11.56 - 21.69, 95% CI) To 5.91 coughs (3.47 - 8.35, 95% CI) (fig. 3.31). Treatment with codeine significantly reduced the ozone-sensitised response by a factor of 3 from 16.6 (11.56 - 21.69, 95% CI) to 6.86 (5.26 - 8.50, 95% CI) (t-test, two-tailed, $p < 0.05$).

The dose of roflumilast (1 mg kg^{-1}) was sufficient to significantly reduce the infiltration of neutrophils into the airways in both rabbits (fig. 3.30) (post-hoc Dunnett's) and guinea pigs (post-hoc Dunnett's) (fig. 3.32).

The dose of salbutamol ($50 \mu\text{g ml}^{-1}$) abolished the methacholine-induced bronchospasm in the guinea pigs but in rabbits the dose of salbutamol ($100 \mu\text{g ml}^{-1}$) only reduced methacholine induced bronchospasm on the R_L to 30.3% (8.1% - 52.5%, CI 95%) above baseline (data not shown).

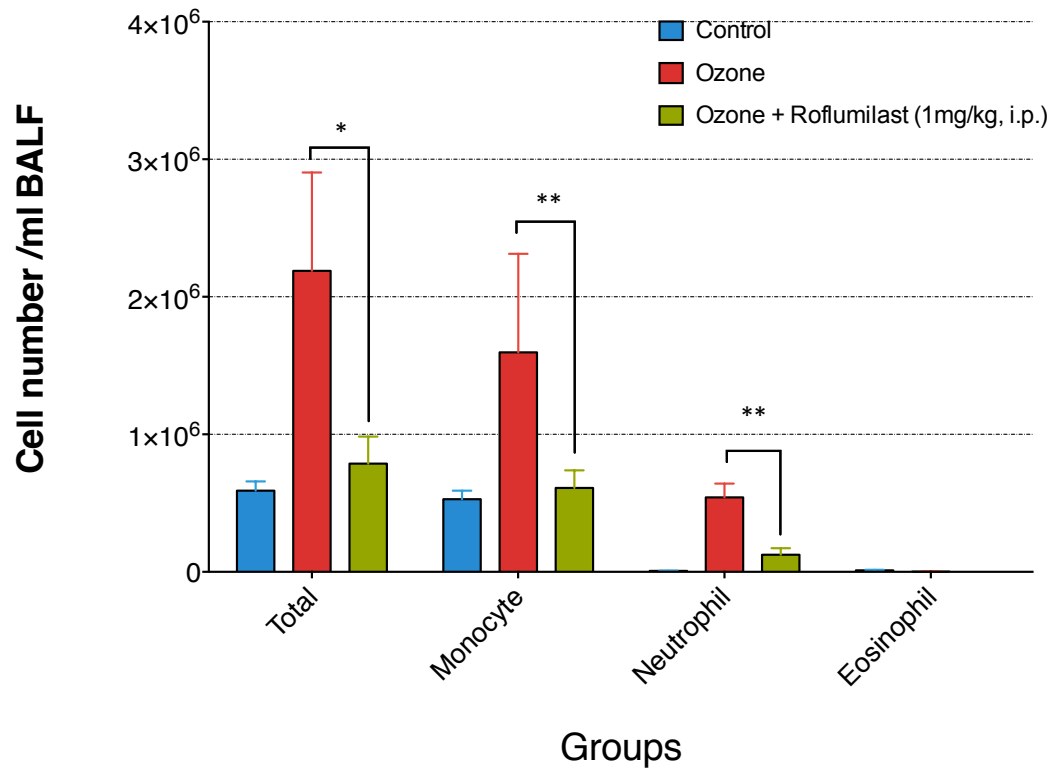


Figure 3.30: The effect of roflumilast on pulmonary leukocytes in rabbits. The error bar represent the mean \pm SEM, n of 16. The connecting lines between bars represents statistical significance, “*” represents $p < 0.05$ and “**” represents $p < 0.01$.

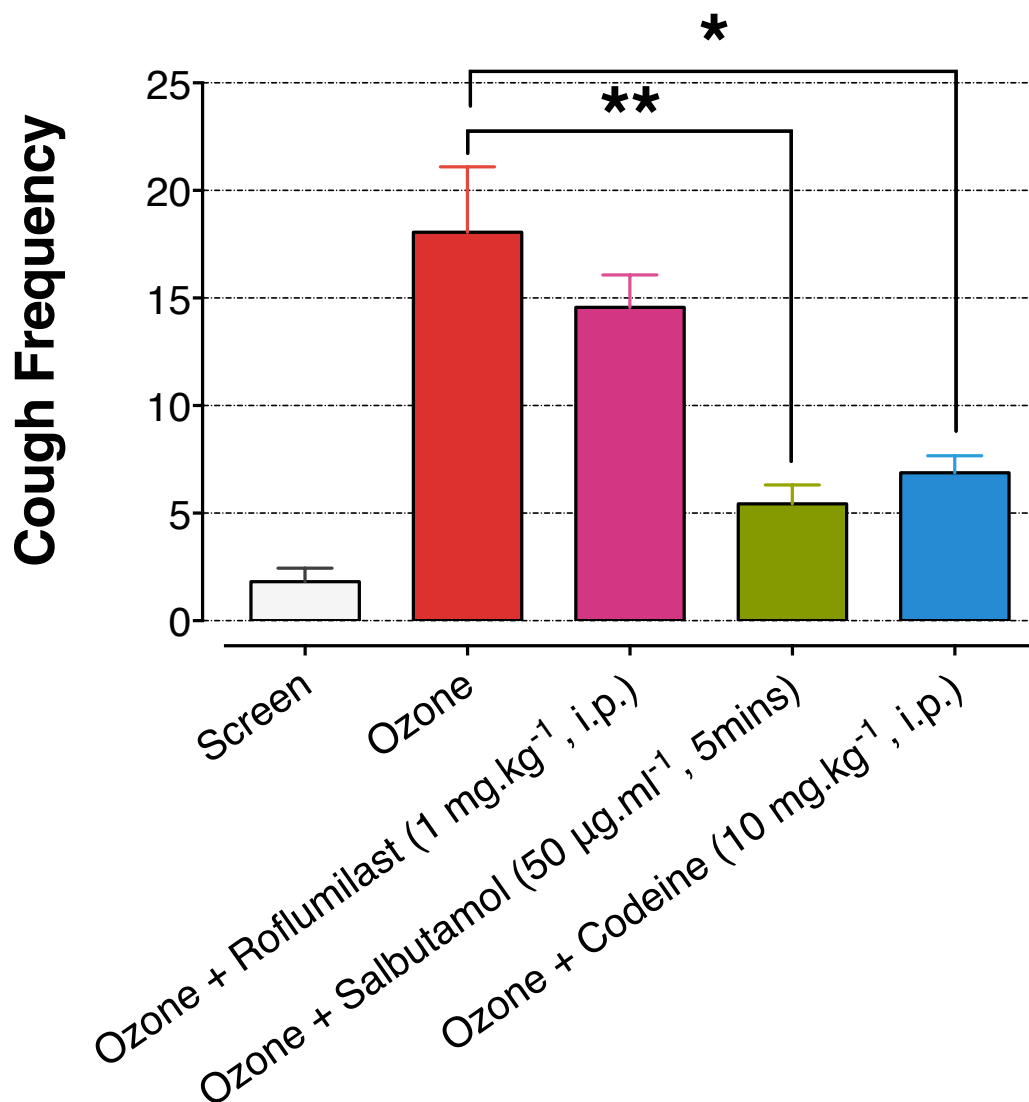


Figure 3.31: The effect of various putative and known antitussives on the cough response in the guinea pigs. The error bar represent the mean \pm SEM, n of 32. The connecting lines between bars represents statistical significance, “*” represents $p < 0.05$ and “**” represents $p < 0.01$.

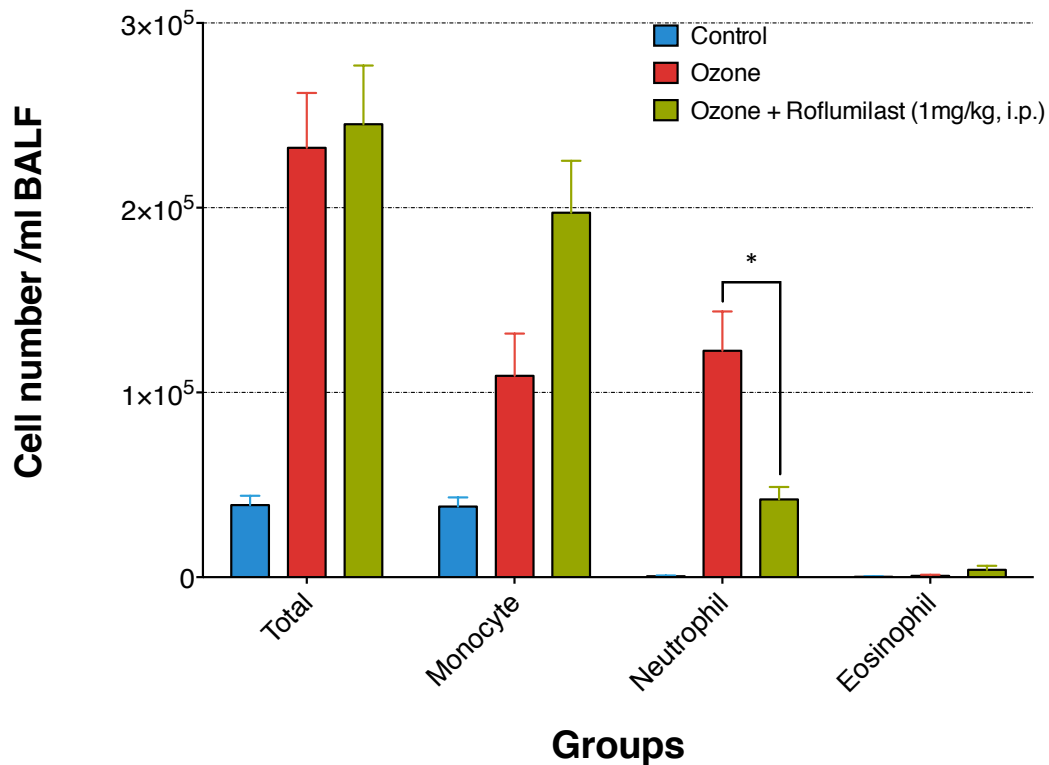


Figure 3.32: The effect of roflumilast on pulmonary leukocytes in guinea pigs. The error bars represent the mean \pm SEM, n of 16. The connecting lines between bars represents statistical significance “*” represents $p < 0.05$.

3.4 Anticholinergic treatment

3.4.1 The effect of anticholinergics on the cough response

In these experiments, the rabbits did not initially respond when screened with citric acid (fig. 3.33). Similarly, there was little response in the negative control for ozone sensitization; when the rabbits were exposed to air with tiotropium bromide treatment. However, with ozone sensitization the mean number of coughs significantly (paired t-test, $p < 0.01$) increased by 8.63 coughs from 0.12 (0.017 - 0.42, 95% CI) to 8.75 (4.03 - 13.5, 95% CI), but this increase was not attenuated by treatment with tiotropium bromide (t-test, $p > 0.05$) fig. 3.33).

Guinea pigs responded poorly on their screen and when exposed to air with tiotropium bromide treatment (fig. 3.34). However, with ozone sensitization the mean number of coughs increased significantly (paired t-test, $p < 0.01$) increased by 16.5 coughs from 1.31 (0.84 - 1.78, 95% CI) coughs to 17.8 (13.2 - 22.4, 95% CI) coughs and this response was significantly attenuated with tiotropium bromide (250 μ M, aerosol 10 mins at 3L.min) reducing the mean response by more than half to 7.38 (4.63 - 10.1, 95% CI) coughs (paired t-test, $p < 0.001$).

Treatment with CHF-6021 produced a similar response in rabbits to tiotropium bromide (fig. 3.35) Ozone sensitization significantly increased the cough response to citric acid by 12.13 coughs (paired t-test, $p < 0.001$) from 1.37 coughs (0.12 - 2.63, 95% CI) to 13.5 (9.36 - 17.64, 95% CI) and, similar to tiotropium bromide, CHF-6021 failed to attenuate the ozone-sensitisation hypertussive response (paired t-test, $p > 0.05$).

Treatment with CHF-5843 produced a similar response in rabbits treated with

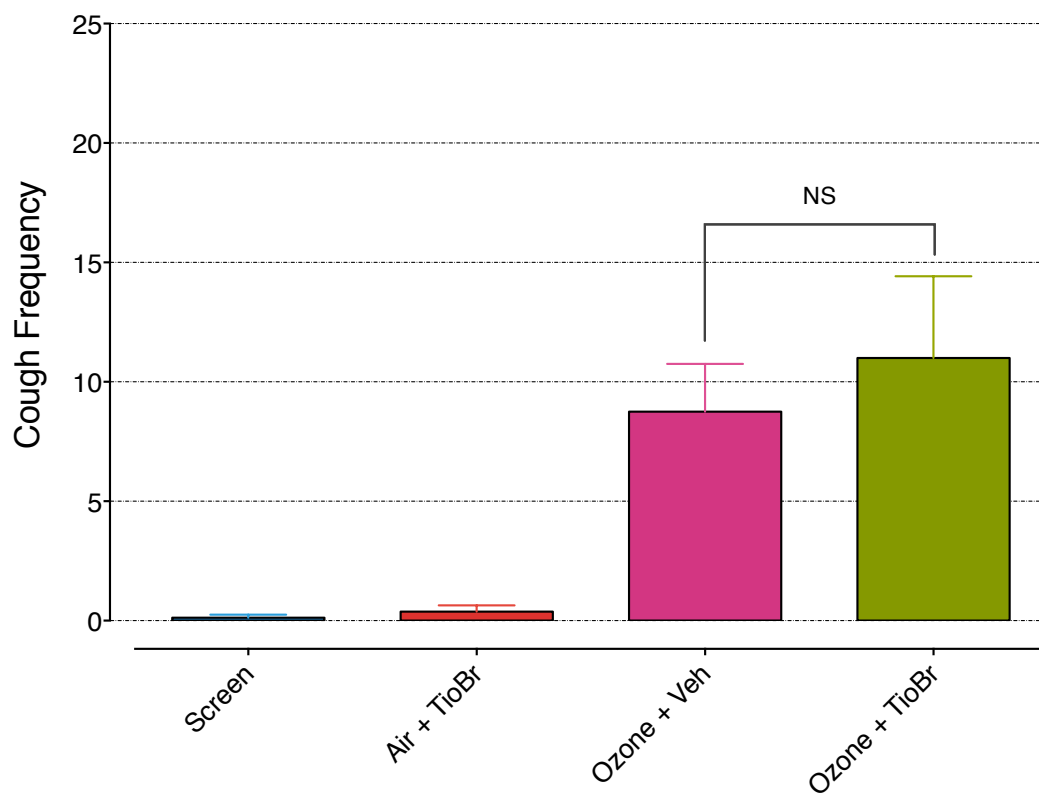


Figure 3.33: The effect of tiotropium bromide on the citric acid induced cough response with and without ozone-sensitisation in the rabbit. Tiotropium bromide was given as an aerosol of a $250\mu\text{M}$ dose dissolved in saline and nebulised using an ultrasonic nebuliser for 10 minutes. Four hours later rabbits were provoked with citric acid (0.8M). The groups represent the treatment group that each animal received and each animal received each treatment group so the bar are paired. The sequence of groups has been normalized but the treatment group that an animal received was randomised to control for sequence effects. The acronym TioBr is Tiotropium Bromide. The error bars represent the standard error of the mean, n of 12. The connecting lines between bars represents statistical significance, “NS” represents No Significance or $p > 0.05$.

tiotropium bromide and CHF-6021 (fig. 3.36). Ozone sensitization significantly increased the cough response to citric acid by 10.6 coughs (t-test, paired, two-tailed, $p < 0.001$) from 1.75 (0.52 - 4.02, 95% CI) to 12.3 (7.32 - 17.2, 95% CI) and,

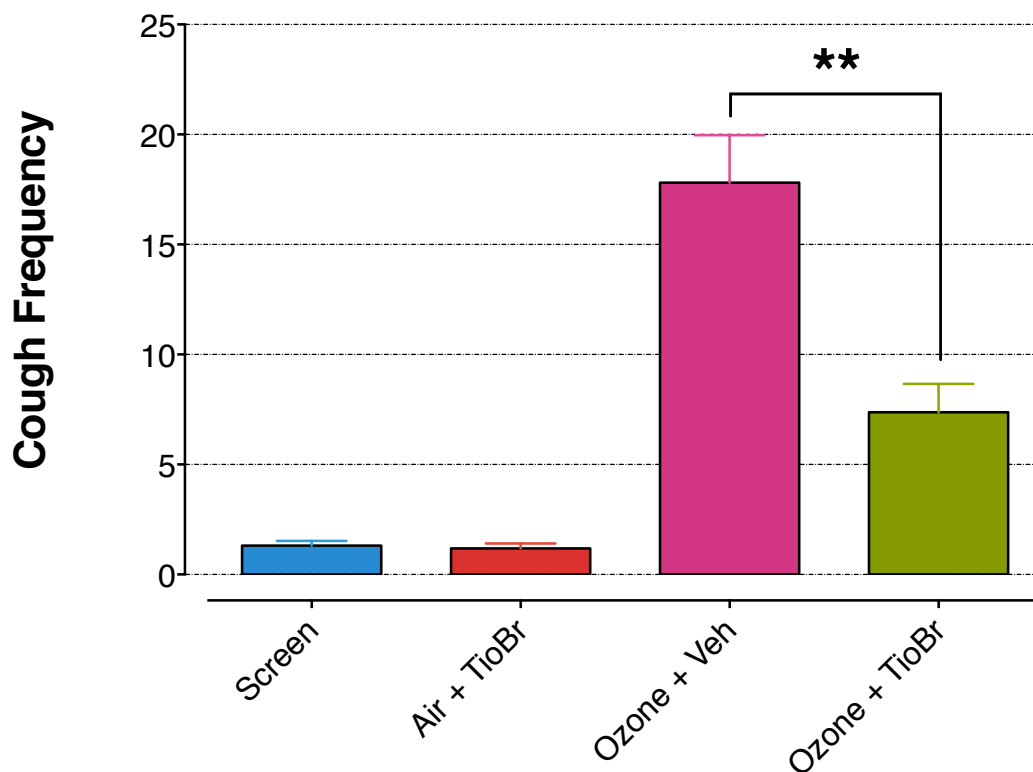


Figure 3.34: The effect of tiotropium bromide on the citric acid induced cough response with and without ozone-sensitisation in the guinea pig. Tiotropium bromide was given as an aerosol of a $250\mu\text{M}$ dose dissolved in saline and nebulised using an ultrasonic nebuliser for 10 minutes. Four hours later guinea pigs were provoked with citric acid (30mM). The groups represent the treatment group that each animal received. The sequence of groups has been normalized but the treatment group that an animal received was randomised to control for sequence effects. The acronym TioBr is Tiotropium Bromide. The error bars represent the standard error of the mean, n of 16. The connecting lines between bars represents statistical significance, “**” represents $p < 0.01$.

similar to tiotropium bromide, CHF-5843 failed to attenuate the ozone sensitised hypertussive response.

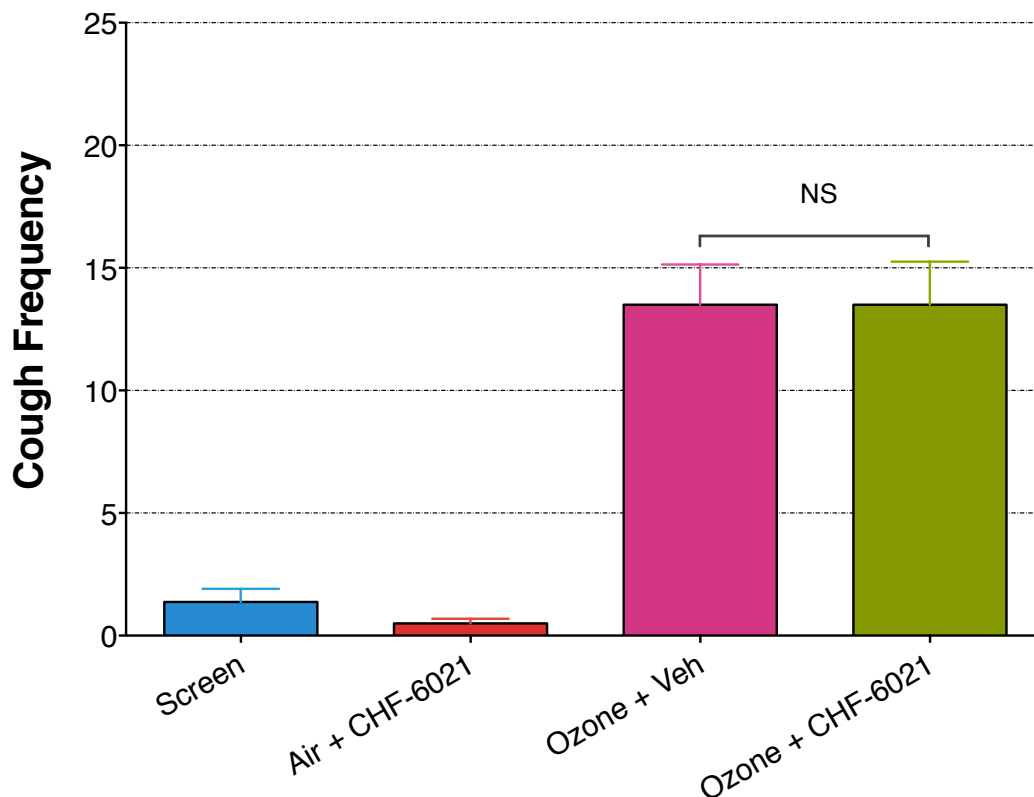


Figure 3.35: The effect of CHF-6021 on the citric acid induced cough response with and without ozone-sensitisation in the rabbit. CHF-6021 was given as an aerosol of a 2.5mM dose dissolved in saline and nebulised using an ultrasonic nebuliser for 10 minutes. Four hours later, rabbits were provoked into coughing with citric acid (0.8M). The groups represent the treatment group that each animal received. The sequence of groups has been normalized but the treatment group that an animal received was randomised to control for sequence effects. The error bars represent the standard error of the mean, n of 8. The connecting lines between bars represents statistical significance, “NS” represents No Significance or $p > 0.05$.

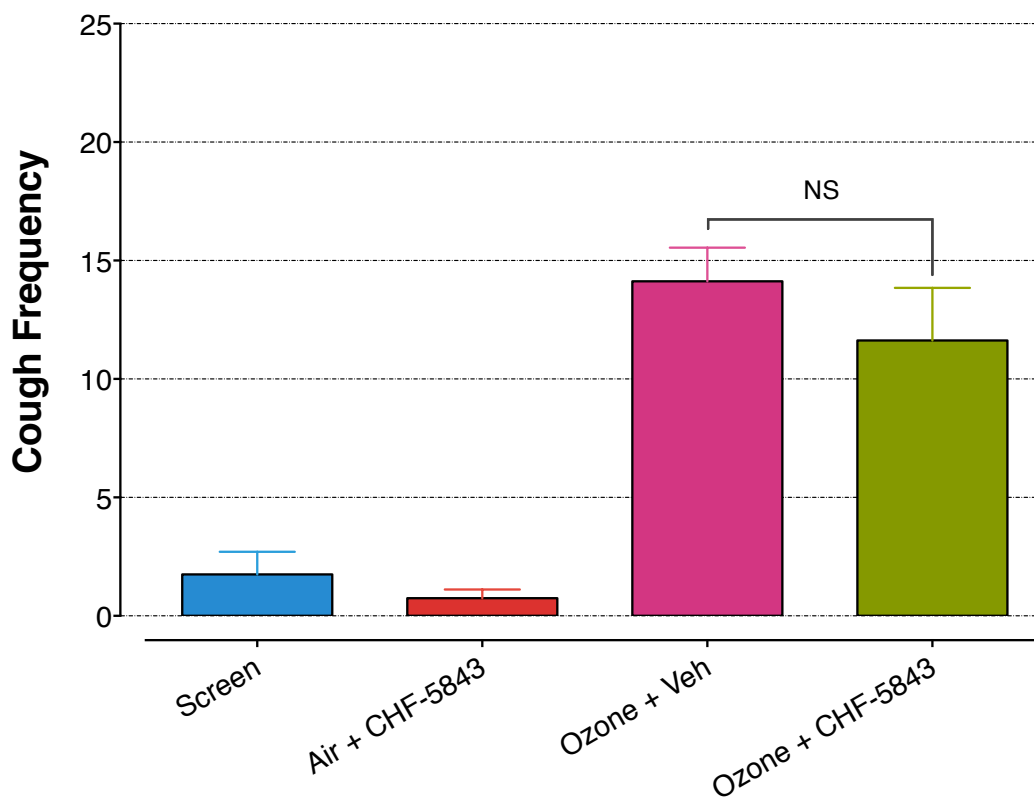


Figure 3.36: The effect of CHF-5843 on the citric acid induced cough response with and without ozone-sensitisation in the rabbit. CHF-5843 was given as a 2.5mM aerosol and nebulised using an ultrasonic nebuliser for 10 minutes. Four hours later rabbits were provoked with citric acid (0.8M). The groups represent the treatment group that each animal received. The sequence of groups has been normalized but the treatment group that an animal received was randomised to control for sequence effects. The error bars represent the standard error of the mean, n of 8. The connecting lines between bars represents statistical significance, “NS” represents No Significance or $p > 0.05$.

3.4.2 The effect of anticholinergics on lung mechanics

Rabbits treated with tiotropium bromide (250 μ M aerosol, 10 minutes) four hours prior to a lung function experiment was sufficient to abolish methacholine induced bronchoconstriction both with and without ozone sensitization (fig. 3.37). A C_{dyn} PC₃₅ or a R_L PC₅₀ was not reached at a maximum methacholine dose of 160mg ml⁻¹.

The anticholinergic treatments were compared to the control group of ozone sensitised methacholine responses illustrated in Figure 3.23 and included on each of the figures for the anticholinergic treatments (Figure 3.37, Figure 3.38 and Figure 3.39) for comparison. To reiterate, the C_{dyn} PC₃₅ for methacholine in the control group without ozone sensitization was, mean \pm CI 95%, 3.71 (2.65 - 3.18, 95% CI) mg ml⁻¹ (fig. 3.23A) and for the C_{dyn} the PC₃₅ to methacholine in the control group with ozone sensitization was significantly lower (paired t-test, $p < 0.02$) at 1.34 (0.91 - 1.77, 95% CI) mg ml⁻¹ methacholine. Similarly, the R_L PC₅₀ for the control group without ozone sensitization was 2.72 (2.08 - 3.34, 95% CI) mg ml⁻¹ methacholine and was lower for the post-ozone control group at 1.32 (0.80 - 1.84, 95% CI) mg ml⁻¹ methacholine but in contrast this was not significant ($p = 0.22$).

Treatment with CHF-6021 (2.5mM aerosol, 10 minutes), four hours prior to the lung function experiment, similar to tiotropium bromide treatment, was sufficient to abolish methacholine induced bronchoconstriction (fig. 3.38). With or without ozone sensitization, a C_{dyn} PC₃₅ or a R_L PC₅₀ was not reached at a maximum methacholine dose of 160mg ml⁻¹.

CHF-5843, 2.5mM given as an aerosol for 10 minutes, four hours prior to the

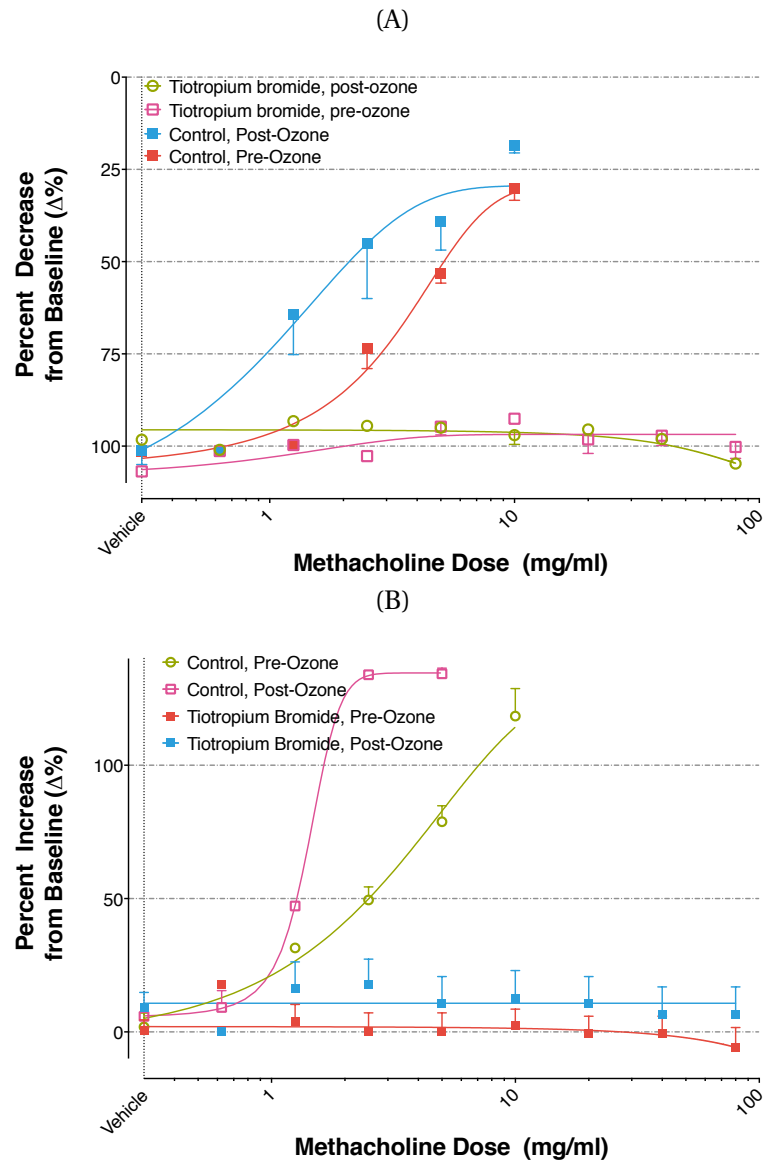


Figure 3.37: The percent change from baseline dynamic compliance (A) and baseline total lung resistance (B) to methacholine in rabbits treated with vehicle or tiotropium bromide. Error bars represent the \pm SEM, n of 12.

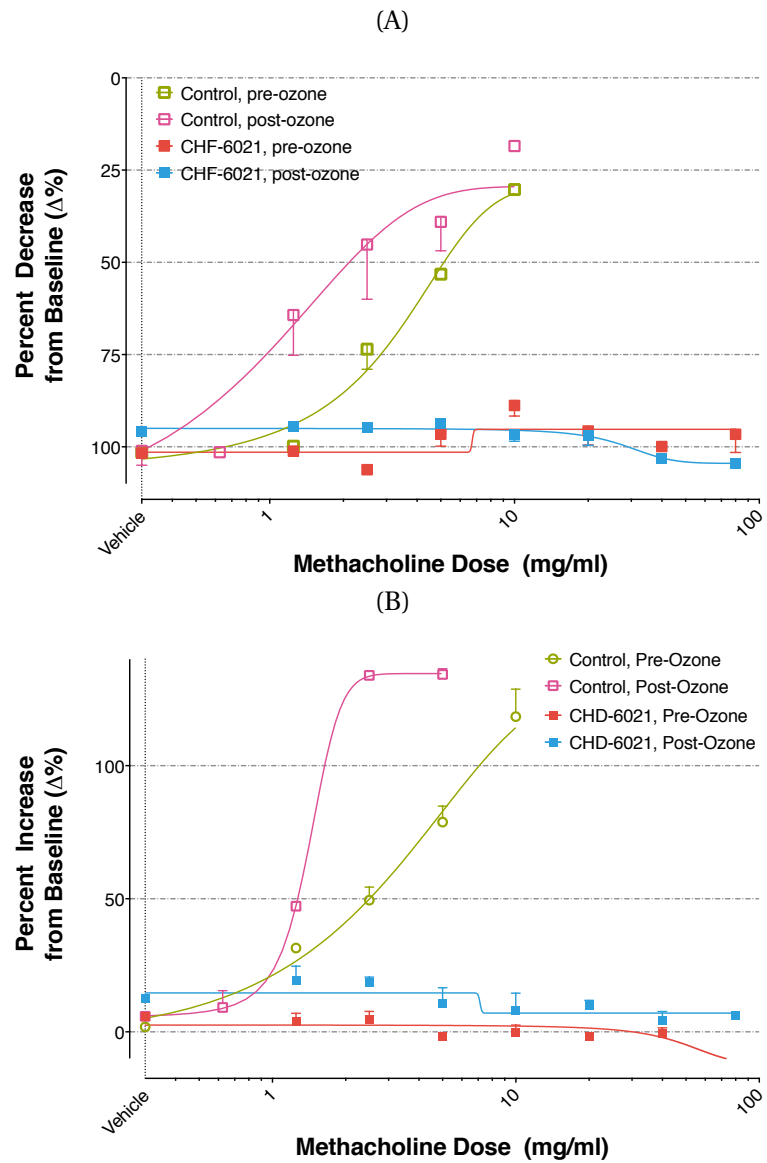


Figure 3.38: The percent change from baseline of dynamic compliance (A) and total lung resistance (B) to methacholine in rabbits treated with vehicle or CHF-6021. Error bars represent the \pm SEM, n of 8.

lung function experiment also caused a rightward shift of the dose response curve for C_{dyn} and R_L , but was not sufficient to block methacholine induced bronchoconstriction (fig. 3.39). The C_{dyn} PC_{35} for methacholine without ozone sensitization in the CHF-5843 group was 76.1 (64.0 - 88.3, 95% CI) $mg\ ml^{-1}$ (fig. 3.23A) but was significantly lower (paired t-test, $p < 0.05$) with ozone sensitization was at 56.8 (50.4 - 68.2, 95% CI) $mg\ ml^{-1}$ methacholine. Similarly, the R_L PC_{50} for methacholine without ozone sensitization was 21.5 (15.2 - 27.6, n of 8) $mg\ ml^{-1}$, but was significantly lower (paired t-test, $p < 0.05$) with ozone sensitization at 34.3 (20.1 - 48.4, n of 3) $mg\ ml^{-1}$ methacholine.

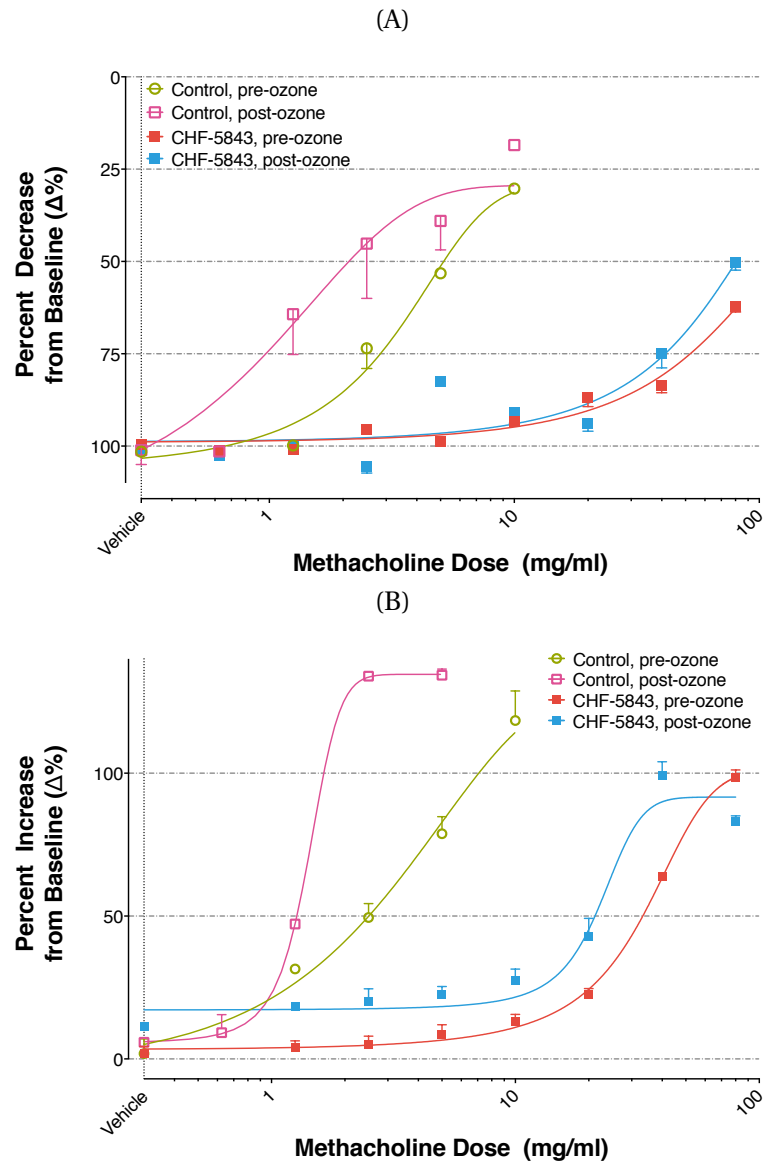


Figure 3.39: The percent change from baseline of dynamic compliance (3.39A) and total lung resistance (3.39B) with and without CHF-5843 treatment. Error bars represent the \pm SEM, n of 8.

3.4.3 The effect of anticholinergics on leukocyte recruitment

Treatment with tiotropium bromide (250 μ M aerosol, 10 mins at 3 L/min) failed to attenuate the ozone-induced increase in neutrophils. There was no significant effect on the total cell number between the untreated (8.7 (3.4 - 20.9, 95% CI) $\times 10^5$ cells/ml) and tiotropium bromide treated (10 (3.1 - 23.3, 95% CI) $\times 10^5$ cells/ml) rabbits exposed to ozone (fig. 3.40). There no significant differences in the differential cell counts between the treated and control group (paired t-test, $p > 0.05$).

Similarly, treatment with CHF-6021 (2.5mM aerosol, 10 mins at 3 L/min) failed to significantly reduce the total cell number the ozone-induced increase in neutrophils. Total cell count was not significantly different between untreated (12.6 (3.9 - 29.3, 95% CI) $\times 10^5$ cells/ml) and CHF-6021 treated (11.4 (4.8 - 27.7, 95% CI) $\times 10^5$ cells/ml) rabbits when exposed to ozone (fig. 3.41). There no significant differences in the differential cell counts between the treated and untreated group (paired t-test, $p > 0.05$).

Lastly and similar to both tiotropium bromide and CHF-6021, treatment with CHF-5843 (2.5mM aerosol, 10 mins at 3 L/min) failed to significantly reduce the total cell number the ozone-induced increase in neutrophils. Total cell count was not significantly different between the treated (12.7 (3.6 - 29.1, 95% CI) $\times 10^5$ cells/ml) and untreated (15.3 (4.22 - 34.8, 95% CI) $\times 10^5$ cells/ml) rabbits when exposed to ozone (fig. 3.42). There was no significant differences in the differential cell counts between the treated and untreated group (paired t-test, $p > 0.05$).

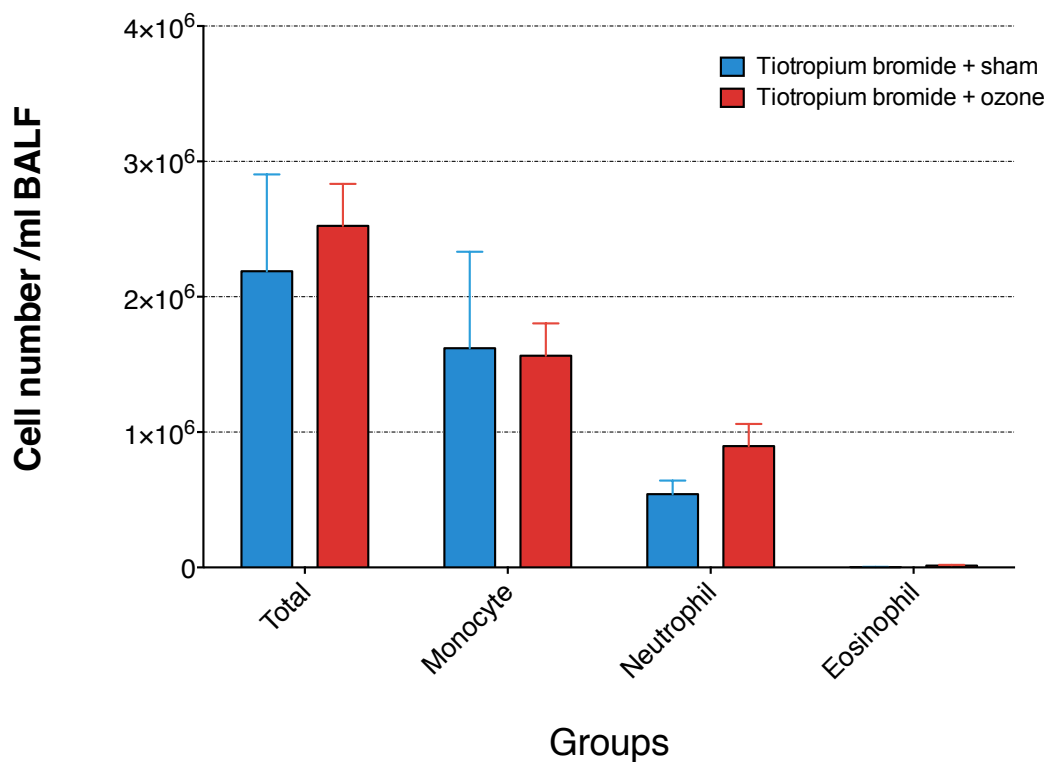


Figure 3.40: The change in total cell count before and after tiotropium treatment in ozone sensitised rabbits. On day 1, rabbits were exposed to 2ppm ozone for 1 hour and then 4 hours later a BAL was performed. Three days later, rabbits were treated with tiotropium bromide, exposed to 2ppm ozone for 1 hour and then 4 hours later they were lavaged again. The bars represent the mean and the error bars are the SEM, n of 8, paired.

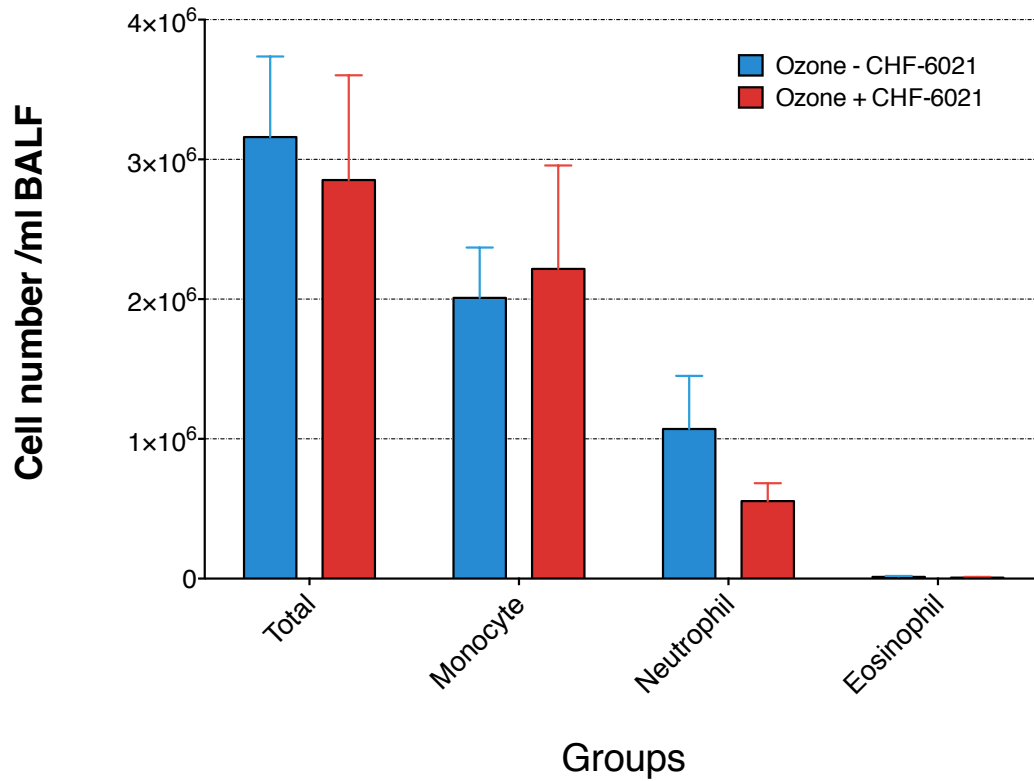


Figure 3.41: The change in total cell count before and after tiotropium treatment in ozone sensitised rabbits. On day 1, rabbits were exposed to 2ppm ozone for 1 hour and then 4 hours later a BAL was performed. Three days later, rabbits were treated with CHF-6021, exposed to 2ppm ozone for 1 hour and then 4 hours later they were lavaged again. The bars represent the mean and the error bars are the SEM, *n* of 8, paired.

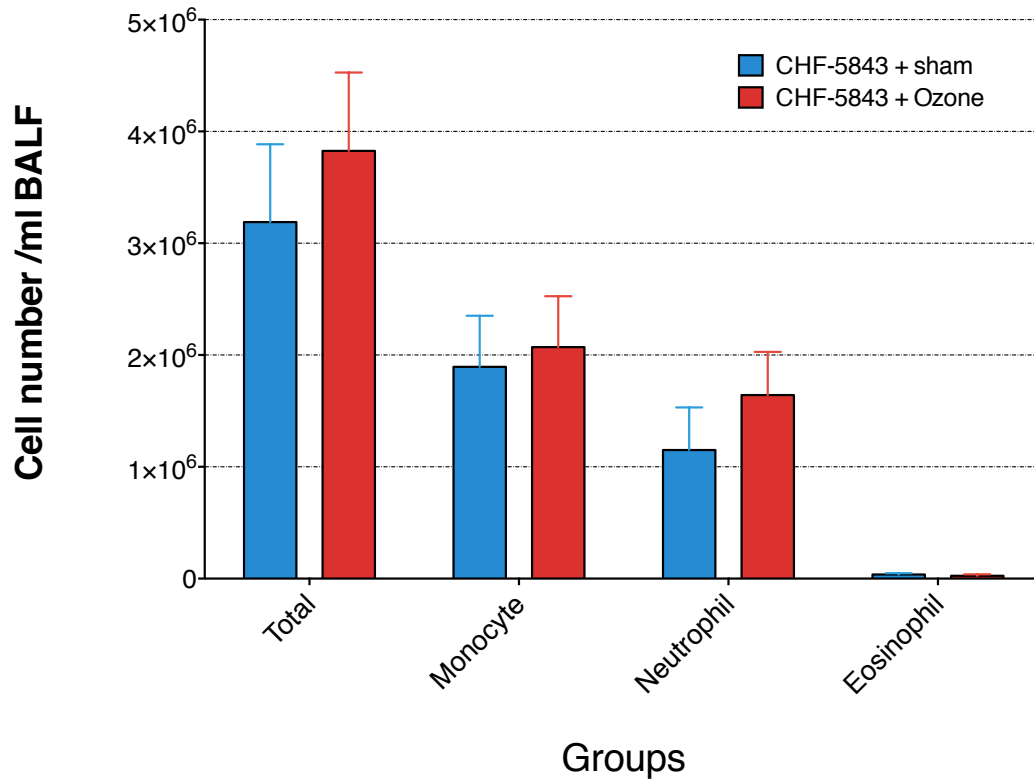


Figure 3.42: The change in total cell count before and after tiotropium treatment in ozone sensitised rabbits. On day 1, rabbits were exposed to 2ppm ozone for 1 hour and then 4 hours later a BAL was performed. Three days later, rabbits were treated with CHF-5843, exposed to 2ppm ozone for 1 hour and then 4 hours later they were lavaged again. The bars represent the mean and the error bars are the SEM, *n* of 8, paired.

3.5 The effect of levodropropizine on the ozone sensitised cough response in guinea pigs

Treatment with a high dose (30 mg kg^{-1} , i.p.), but not a low dose of levodropropizine (10 mg kg^{-1} , i.p.), 30 minutes before ozone sensitization significantly reduced the ozone sensitised cough response to citric acid (Repeat measures ANOVA with post-hoc Tukey's test) from 8.86 (2.63 - 15.1, 95% CI) to 2.63 (0.84 - 4.41, 95% CI) (fig. 3.43). Treatment with chlorpheniramine did not significantly reduce the ozone sensitised cough response.

Concurrent treatment of levodropropizine (30 mg kg^{-1}) with chlorpheniramine (30 mg kg^{-1}) failed to demonstrate any significant attenuation of the ozone sensitised cough response (fig. 3.43).

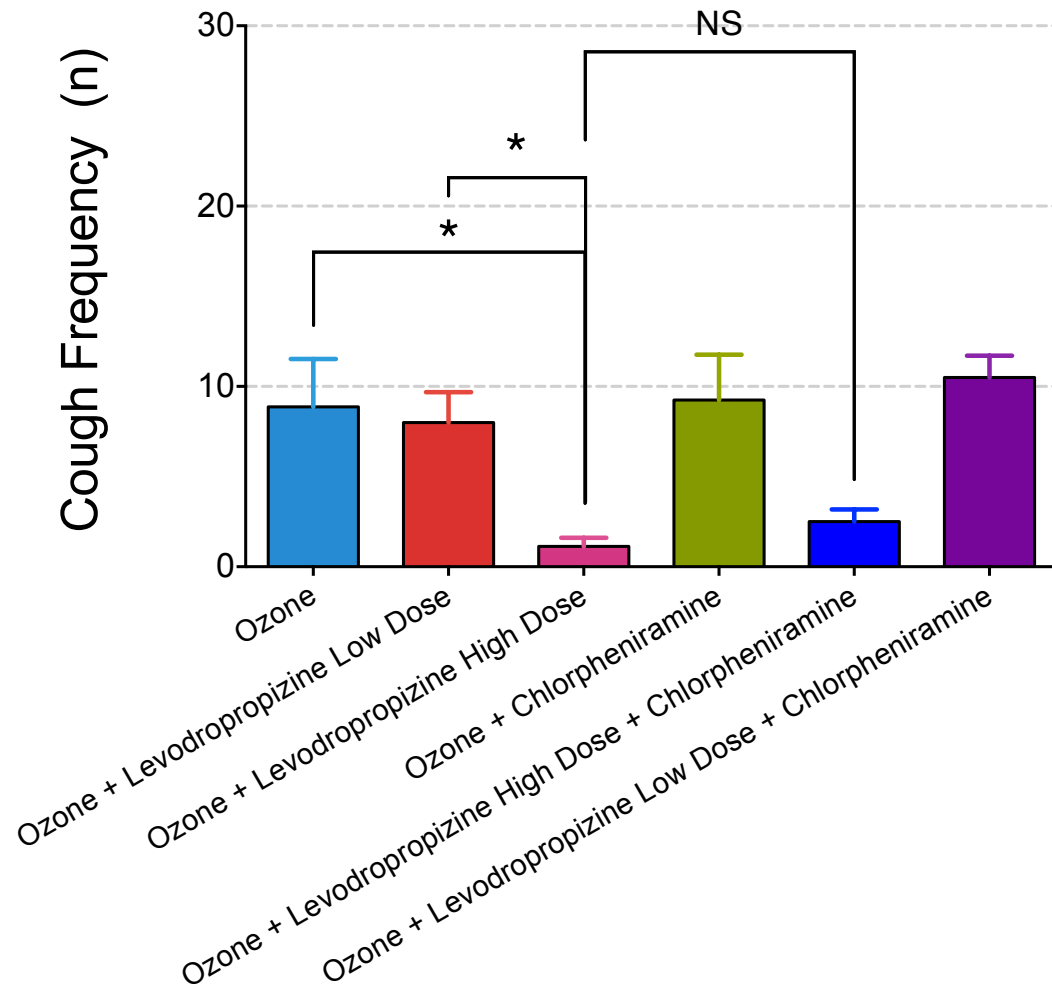


Figure 3.43: The effect of levodropropizine treatment with and without chlorpheniramine treatment on the ozone sensitised cough response in guinea pigs. The abbreviation “Levo” refers to levodropropizine, error bars represent the \pm SEM. n of 16, paired. The connecting lines between bars represents statistical significance, “NS” represents No Significance or $p > 0.05$ and “*” represents $p < 0.05$.

Chapter 4

Discussion

4.1 Objective measurement of cough

Sensitised, hypertussive animal models of cough may provide a better model to test, screen and validate putative antitussives providing a valuable tool in pre-clinical pharmacology (Mackenzie *et al.*, 2004).

Establishing a hypertussive animal model of cough first requires effective classification of a cough event. Fortunately, both coughs and sneezes can be easily discriminated from background noises because they have a characteristic explosive sound, they are relatively loud in amplitude and last for around 60 - 500msecs. It is difficult, however, to separate cough sounds from sneeze sounds and this is a problem that has been highlighted in reviews on the subject (Morice *et al.*, 2007). This present study confirms these issues and establishes that neither the interval nor the amplitude of a sneeze or a cough can categorically distinguish one event from the other.

The problem discriminating cough sounds from sneeze sounds arises largely from the fact the information of that sound is not in the time domain and thus the shape, thresholds and peaks of the audio signal do not indicate what the sound is but only how loud it was, how long it went on for and when it occurred. This present study used the frequency spectrogram to improve the determination of the start and end of an audio event because the edge of the frequency change is sharper than an inflection of the audio signal in the time domain. In certain canonical cases, the frequency spectrum of a cough is visibly different from a sneeze. However, human observation of the spectrogram is not enough to discriminate coughs from sneezes and a more sensitive method of discrimination would need to be attempted in future to objectively measure cough from the frequency spectrum. This present study has yielded a well annotated, machinable dataset of annotated coughs and sneezes with the accompanying metadata about the sequence of experiments that a given individual subject had. It is hoped in future, that we may develop a method to classify cough from sneezes and perhaps different classes of cough from one another.

In this present study, both commercial cough classification systems, the EMKA cough analyser (EMKA, PZ100W-Z) and the Buxco cough analyser (Buxco, FinePointe Series), use the interval and the amplitude of the sound to classify the cough signal. The EMKA cough analyser classifies a cough as the simultaneous increase of the plethysmography chamber pressure and the sound above a user-defined threshold within a given time period. The Buxco cough analyser merely classified the change in the plethysmography chamber

pressure using a method unspecified in their manual. However, based on observing the classification viewer in Buxco's FinePointe software, it appears to be analysing the slope of the signal from the peak of the event to the trough by linear regression. Essentially, both systems were adequate for automatically classifying the cough in guinea pigs because they appeared to only cough to citric acid not sneeze. Other groups have claimed to be able to hear when a guinea pig is sneezing (Xiang *et al.*, 1998; Leung *et al.*, 2007), but I was not confident that this was possible in our guinea pigs. However for rabbits, the automatic classifiers very frequently mistake sneezes for coughs and no attempt is made by these systems to separate the two. Fortunately, it is possible to discriminate a cough from a sneeze by listening to a rabbit coughing and determine a cough from a sneeze manually; this is what we recommend other groups to do.

4.2 Ozone sensitisation of the citric acid induced cough

Ozone is a powerful sensitization agent and has previously been shown to enhance the cough reflex 16-fold in rabbits and in many cases sensitise a rabbit from being unresponsive to citric acid to having a hypertussive response (Adcock *et al.*, 2003). This thesis reproduces what Adcock *et al.* (2003) demonstrated and further contributes to the evidence that ozone is an effective sensitization agent by showing that ozone can consistently and reliably increase the cough response of both rabbits *and* guinea pigs; shifting the cough dose response curve to citric acid leftward by between 0.5 to 1 log units. Similar to

Adcock *et al.* (2003), ozone exposure sensitised rabbits to cough from no response to a discernible response. However, in contrast to Adcock *et al.* (2003) many rabbits (84%) would cough to citric acid during screening, but very poorly on subsequent exposures, whether that was the next day or whether it was week after the initial insult. The difference in the response may be due to a systematic error, differences between the animal populations used or minor variations between our protocols and Adcock *et al.* (2003). The poor response in subsequent exposures post-screening could either be attributed to tachyphylaxis, suggested by Figure 3.11 for rabbits and Figure 3.13 for guinea-pigs, or that the first time that rabbits and guinea pigs are exposed (screened), the animals are not used to citric acid exposure and thus cough but subsequently are used to citric acid exposure and do not cough - either it's true desensitisation or a behavioural artifact. It is probable that in the guinea-pig that it is tachyphylaxis, Lalloo *et al.* (1995) demonstrated that the response to citric acid returns after 7 days in the guinea pig. However, in the rabbits the response to citric acid didn't return to screening levels even after 28 days (fig. 3.16) and this compares favourably with Adcock *et al.* (2003) observation that rabbits have a poor response to citric acid and thus the screening response could be a behavioural artifact.

Extending the work of Adcock *et al.* (2003), ozone can be used to sensitise rabbits on multiple occasions in a reliable and reproducible manner meaning that individually-controlled cross-over experiments can be conducted. This is an important concern for citric acid induced cough experiments because cough responses are idiosyncratic and thus highly variable, and by controlling for each

animal individually reduces both the number of animals and number of trials required. In addition, drugs that have a weak affect on the cough response can be repeated a number of times until the necessary statistical power is satisfied.

It was interesting to observe that of those rabbits that respond without sensitization to citric acid approximately 10% of rabbits had a greatly exaggerated response and this accounts for the large variation in the citric acid dose response curve. However, this exaggerated response was not seen in subsequent exposures to citric acid nor present when the animals were exposed to ozone and provides further evidence that the screening response is a behavioural artifact because it could be expected that if these rabbits were hypersensitive then it may have an effect on the degree of tachyphylaxis. It is of note that using a low dose, for instance an EC₂₀ dose, of citric acid with ozone sensitization the animals responded in a more reliable manner; there is significantly smaller variation in the number of coughs reported. Ozone sensitization is not species specific to rabbits and in this study it is clear that ozone can sensitise guinea pigs just as effectively if not more effectively than rabbits. This also suggests that ozone is acting on a common mechanism in both species. Lastly, it was observed that often the sound of the ozone sensitised cough was louder, drier and shorter this was not always the case but bears similarity with how the sound of normotussive cough changes in the disease state in humans.

The ozone sensitised cough response compares favourably with what has been seen in allergen sensitised models. In the dog, the cough response doubled (10 ± 2 coughs to 20 ± 3 coughs) from baseline when sensitised with an allergen

(House *et al.*, 2004) and, similarly, in the guinea pig a doubling (1.9 ± 0.3 to 4.2 ± 0.8 ; 10^{-6} M capsaicin) or tripling of the cough frequency at a higher dose (9.1 ± 0.8 to 33.1 ± 3.0 ; 10^{-1} M capsaicin) can be expected (Liu *et al.*, 2001). Sensitisation with tobacco smoke in guinea pigs also leads to roughly a doubling in the cough response, (24 ± 4 coughs vs. 12 ± 2 for air-exposed animals) (Lewis *et al.*, 2007). In our experiments, the cough frequency increased by a factor of 4 (1.4 ± 1 to 6.3 ± 1.5 coughs; 0.4M citric acid) or a factor of 8 at a higher dose (0.16 ± 0.13 to 8.15 ± 1.3 ; 0.8M citric acid) in rabbits - it is of note that the response is roughly half the magnitude observed in Adcock *et al.* (2003) original experiments (0.18 ± 0.18 to 15.9 ± 4.93 coughs) but similar in that it is an increase from, essentially, no response to a strong response. The magnitude of difference between baseline and sensitised response suggest that the ozone sensitised rabbit is a more sensitive model for differentiating between the healthy and disease state. Further, ozone sensitizes the cough acutely (4 hours) as opposed to the allergen sensitized models (27 to 30 days) and therefore offers a simpler and less time-consuming model of hypertussive cough.

4.3 Cross-over designs and the ozone sensitised cough

Cross-over designs are a powerful way to control for the individual bias on the cough response and increase the number of samples for each treatment group by reusing animals. There are a number of reasons we chose to use cross-over designs and these were predicated on early experiments performed. Firstly it

was observed that the dose response to citric acid was highly variable for both rabbits (Figure 3.10) and guinea pigs (Figure 3.12) on their first naïve exposure to citric acid. Secondly, repeated daily dosing with citric acid and repeated weekly dosing failed to elicit the same response that the animals first achieved on their screen. Thirdly, it was desirable to compare “hypertussive” responses to normotussive responses. This made cross-over experimental designs particularly useful as it meant that animals could be “screened” for their naive response before establishing a normotussive response, there was robust control for individual bias rather than using a control group average and normotussive and hypertussive responses could be matched pairwise for each subject. In later experiments, it meant that each treatment group was individually matched to their negative and positive controls. Lastly, cross-over designs, while popular in clinical antitussive research, are not particularly popular in preclinical antitussive research so this was an opportunity to attempt something novel.

Using the cross-over design requires more attention to the experimental plan and performing more complex statistical analyses. Firstly, the experiments must be balanced so that they are uniform within periods and uniform within sequences. Balancing an experiment so that it was uniform within periods wasn't particularly challenging in this particular thesis. Firstly, none of the treatment groups were expected to stop cough responses because the treatments were non-lethal and the effects of treatment are reversible. This meant that all subjects could be included in all periods. Secondly, hypertussive cough responses were recorded using a low dose of citric acid where the variance in the responses between unsensitised and sensitised cough responses

was lower which meant that the effects of confounding variables would have been more obvious. Balancing an experiment so that it was uniform within sequences was fine for simpler 2x2 and 3x3 experimental designs where there are 2 ($2! = 2$) and 6 ($3! = 6$) possible combinations but more complicated when screen an number of putative antitussive treatments as each subject required 6 periods and thus there are 720 possible combinations; screen, negative control (air), positive control (ozone), salbutamol, roflumilast and codeine. The number of combinations was reduced by assuming that screen, negative control, positive control had no affect on the cough response and this was supported by the earlier experiments and the statistical analysis thereof. This is, however, a flaw of our experimental designs as it is not as robust as appropriate powering of all possible sequences but this was considered more favourable than not being able to individually control for each subject. The 7 day interval was considered sufficient to control for carryover effect experimentally as this was 3 times the half life or greater; no effect of carryover was observed.

Mixed effect linear modelling was used as the statistical model to test for effects of sequence (carryover) and period. Understanding how mixed effect linear modelling works is no more challenging than understanding the student's t-test or ANOVA, but it is a statistical model that I was less familiar with. Implementation of mixed effect linear modelling is certainly more challenging as it often requires more complex and less user friendly software such as SAS, SPSS, R or statsmodels rather than Excel or GraphPad. Further, the software expects that the user has formatted the data in a particular way and unless the user knows how to generate a sequence or period vector then these must be

entered manually before the test can be run. This also leads to a secondary issue whereby the dataset becomes a heterogeneous collection of your dependent variable with various covariate rather than a simple homogeneous collection of your dependent variable. The effect, however, is that if user keeps their data in a normalised fashion then applying more complex statistical techniques is made much easier.

4.4 The mechanism of action of ozone sensitised cough

This study has attempted to identify whether ozone acts on a specific endogenous target and the evidence suggests that ozone acts in a non-specific manner. Firstly, a chronic capsaicin exposure experiment was used to determine whether ozone sensitisation is transduced by airway sensory nerves and this was demonstrated by using chronic capsaicin exposure to desensitise neuropeptide-containing airway sensory nerves (See Section 2.9.3). Capsaicin desensitises the airway sensory nerves by phosphorylation of TRPV1 leading to desensitisation mediated by a cAMP-dependent protein kinase (PKA) signal pathway (Mohapatra and Nau, 2003), it causes the depletion of sensory neuropeptides and damages the nerve causing the loss of TRPV1 channels from the airways (Watanabe *et al.*, 2006). Chronic capsaicin desensitised rabbits, illustrating that ozone sensitization is transduced by peripheral airway sensory nerves because desensitising the TRPV1 containing sensory nerves blocks the ozone sensitised citric acid induced cough response (Lundberg and Saria, 1983;

Sekizawa *et al.*, 1996; Tanaka and Maruyama, 2005). Secondly, evidence from Taylor-Clark and Udem (2010) demonstrated that ozone binds to TRPA1 receptors and that TRPA1 receptors can be found in association with airway C-fibres. However, the TRPA1 antagonist HC-030031 failed to attenuate ozone sensitization in rabbits and a different TRPA1 agonist failed to sensitise rabbits to citric acid in the way ozone does. Therefore TRPA1 is unlikely to be a causal mechanism by which ozone sensitise the airways to citric. This observation was also confirmed in guinea pigs as they will cough when exposed to cinnamaldehyde and that this cinnamaldehyde-induced coughing can be antagonised with HC-030031, an observation seen elsewhere (Birrell *et al.*, 2009; Geppetti *et al.*, 2010; Silva *et al.*, 2011). However, ozone sensitised hypertussive responses to cinnamaldehyde were not significantly attenuated by HC-030031. Thirdly, ozone sensitization had an acute effect on the cough response so it is unlikely that the ozone is sensitising the airways by non-specific damage to the epithelia - damage that would initiate an inflammatory response and thus could sensitise the airways to cough - because the time-course of ozone sensitisation is too short. The demonstrable rise in total leukocytes numbers in the BALF and the neutrophilia demonstrated in both guinea pigs and rabbits occur 4 hours after ozone sensitization, but are not present when the cough response is measured 1 hour after ozone challenge suggesting that this immune response is secondary to the mechanism by which ozone is sensitising the cough response. However, the Bronchial Hyperresponsiveness (BHR) to methacholine caused by ozone sensitization only appears to manifest 4 hours after ozone exposure suggesting that the BHR was associated with the inflammation and

neutrophilia. There is a well established association between BHR, lung inflammation and neutrophilia (Xiu *et al.*, 1995; Grootendorst and Rabe, 2004) as well as an association between ozone exposure and BHR (Koto *et al.*, 1997). Antagonising the production of IL-8, a chemotactic factor for neutrophils significantly inhibits BHR in the guinea pig (Xiu *et al.*, 1995), similarly, Leukotriene B₄ antagonists can inhibit BHR by reducing the adhesion and activation of leukocytes (Grootendorst and Rabe, 2004). However, a specific anti-Cytokine-induced neutrophil-chemoattractant (CINC) monoclonal antibody in the rat blocked neutrophilia without affecting BHR (Koto *et al.*, 1997) implying that neutrophilia is not necessary to cause BHR in an ozone-induced inflammatory model. (Crimi *et al.*, 1998) supports this by demonstrating that macrophages are more strongly correlated with BHR than neutrophils are in humans. It is likely therefore that the mediators released during the inflammatory response to ozone can cause BHR, but that the mechanism of action isn't specific to neutrophils or macrophages.

the cough response. Also which sub-type/s maybe involved in the sensitisation evoked by ozone?

Sensory airway nerves are evidently playing a role in both the stimulation of cough and the sensitisation of the airways to cough in both the rabbit and the guinea pig subjects, but the relative importance of which airway nerves and which receptors is unclear. C-fibres and A δ -fibres are the two prominent nerve subtypes that associate with a variety of receptors that respond to many of the known tussigenic agents so it likely that one of more of these targets is involved. In this thesis we predominantly used citric acid to stimulate cough and

capsazepine, a TRPV1 antagonist, can block citric acid induced cough in guinea pigs (Lalloo *et al.*, 1995). TRPV1 is found predominately on C-fibres, but is also present on A δ -fibres (Watanabe *et al.*, 2006; Delescluse *et al.*, 2012) and perhaps the reason why citric acid is an effective tussive agent is that it stimulates both of these afferent pathways simultaneously. Similarly, our study failed to demonstrate a specific endogenous target that ozone was acting upon and this may be a reflection of the fact that ozone doesn't act on one specific target, but more than one. This is an entirely reasonable proposition; ozone is a small, simple inorganic chemical that acts as a powerful and broad oxidative agent so it is highly unlikely that there is a specific endogenous target for ozone. It is likely that TRPA1 is involved because it has been shown that ozone binds to TRPA1, but because TRPA1 alone can't sensitise a rabbit to cough then there may need to be an additional signal to explain how ozone sensitises the airways to cough (Taylor-Clark and Udem, 2010). Ozone causes cell damage so that additional signal could be one or more early-response inflammatory mediators such as Platelet Activating Factor (PAF), TNF α or IL-6 which released early phases of an inflammatory reaction and peak at around 60 minutes post-insult (Klosterhalfen *et al.*, 1992) which coincides with the time the citric acid cough challenge was conducted.

4.5 Validating and screening antitussive drugs with the ozone sensitised model of cough

The first four aims of this study were to establish how to best objectively measure cough and investigate the ozone sensitised citric acid induced model in the rabbit and guinea pig - with these models established known and putative antitussives were investigated. Codeine was chosen as a positive control for antitussive activity and in both rabbits and guinea pigs it reduced the mean cough response by 42% (13.03 - 97.03, 95% CI) and 53.4% (27.4 - 82.6, CI 95%), respectively. It suggests that codeine at the dose used, 6 mg kg⁻¹ i.p., was sufficient to block the hypertussive cough but not abolish the cough reflex entirely and no sedation was evident. The relative reduction in the cough number is similar to that reported by Karlsson *et al.* (1990) and Kotzer *et al.* (2000). Qualitatively, there was no difference in cough sound in the ozone-sensitised rabbits whether they received codeine or not. Codeine was effective at attenuating the cough response and was a useful positive control in both the rabbit and guinea pig preclinical models.

In an adjunct set of experiments, ozone sensitised guinea pigs were treated with levodropropizine at a low dose of 10 mg kg⁻¹, i.p, and a high dose 30 mg kg⁻¹. Levodropropizine is an opiate with a similar efficacy to dextromethorphan (Catena and Daffonchio, 1997) and in our study at the higher dose, 30 mg kg⁻¹ i.p., significantly reduced ozone sensitised cough by a similar magnitude to codeine, 6 mg kg⁻¹ i.p.. It is further evidence that the opiate class is effective as both antitussives and anti-hypertussives. The guinea pigs treated

with levodropropizine were also crossed-over with a high dose of chlorpheniramine (30 mg kg^{-1}). The aim was to assess the complementary effect of an anti-decongestant and sedation (chlorpheniramine) with levodropropizine. It is a pertinent question because chlorpheniramine is commonly added to OTC cough formulations (Schroeder and Fahey, 1996) and the therapeutic action is both as an anti-decongestant (Morice and Abdul-Manap, 1998) and as a sedative (Bolser, 2008; Padma, 2013). However, there is concern about drug interactions such as potentiation of the appetitive effects of dihydrocodeine (Suzuki *et al.*, 1990) and, more severely, generalized convulsions and intoxication (Murao *et al.*, 2008). In our study, chlorpheniramine with or without concurrent levodropropizine treatment did not significantly attenuate the ozone sensitised cough response. This was a pilot study so conclusions are limited, but it is a cause for concern if there isn't significant improvements in primary efficacy with this drug combination of chlorpheniramine and levodropropizine because of the aforementioned drug interactions and that it is available as a self administered OTC formulation.

Treatment with roflumilast had a similar effect in both guinea pigs and rabbits in this study and while, in both animal models, roflumilast demonstrated no significant effects on the cough response, roflumilast caused a significant decrease in neutrophilia in both animals and a significant decrease in total cell numbers in guinea pigs. This suggests that neutrophilia is not particularly important in the ozone sensitised cough response and that the mechanism of action of ozone sensitization is not dependent on the neutrophilia that results from ozone exposure. This is in contrast to ovalbumin

sensitised guinea pigs that demonstrate significant inhibition of the cough reflex when treated with a selective PDE4 inhibitor, SB207499 (Hj *et al.*, 2004). This does not rule out the possibility that roflumilast, and thus PDE4 inhibition, isn't a good target for cough as PDE4 inhibition is an effective anti-inflammatory, as demonstrated by our data and evident in other studies (see Section 1.5.4). It simply implies that PDE4 inhibition is not particularly effective as an acute non-specific antitussive but it could be used to target inflammatory symptoms that are often concomitant to cough symptoms.

Treatment with salbutamol had contrasting effects in the guinea pigs and rabbits. In guinea pigs, salbutamol ($50 \mu\text{g ml}^{-1}$, aerosol) significantly reduced the ozone-sensitised citric acid cough response by 44.8% (8.96 - 80.7, CI 95%) however in rabbits salbutamol demonstrated no significant effect on the cough response. It is known that citric acid can induce bronchoconstriction and that the effect can be mediated by bradykinin B_2 receptors and NK_2 receptors (Ricciardolo *et al.*, 1999) and it is likely that functional antagonism of airway smooth muscle contractions and/or oedema is the explanation for the preclinical false-positive results of salbutamol as an antitussive agent (Karlsson *et al.*, 1999). Our expectation is that if bronchodilation has a similar effect in both guinea pigs and rabbits then we would have expected to have seen a small decrease in the cough response to correspond with the reduced efficacy of salbutamol in rabbit rather than no discernible response. One explanation is due to a species difference in the expression of β adrenergic receptors subtype. Rabbits express a greater ratio of β_1 to β_2 receptors (Rugg *et al.*, 1978) and salbutamol is 2818x more selective for β_2 than β_1 (Baker, 2005). Therefore the

reduced availability of β_2 receptors to salbutamol may explain the corresponding reduction in efficacy in the rabbit. We controlled for this in our study by assessing the effectiveness of salbutamol to reverse the methacholine-induced bronchoconstriction in four rabbits. All four of the rabbits responded to salbutamol, 100 μgml^{-1} aerosol 20 seconds, and salbutamol reduced the effect of methacholine-induced bronchoconstriction on the R_L to 30.3% above baseline from a mean peak response of 120.2 (85.5 - 154.5, 95% CI) $\text{cm H}_2\text{O.s.L}^{-1}$ to 78.1 (57.0 - 99.2, 95% CI) $\text{cm H}_2\text{O.s.L}^{-1}$ where the baseline R_L was 62.3 (48.1 - 76.7, 95% CI) $\text{cm H}_2\text{O.s.L}^{-1}$. In contrast, salbutamol, 50 μgml^{-1} aerosol 20 seconds, abolished the methacholine-induced bronchospasm in the guinea pigs. These findings suggest two things; first, because salbutamol attenuates both the hypertussive and normotussive citric acid cough response in guinea pigs (Ricciardolo *et al.*, 1999) it suggests that bronchoconstriction is important in exacerbating cough responses in the guinea pig. Second, it suggests that the rabbit is a good animal model for cough for testing drugs that have primary or secondary effects on bronchodilation because it is insensitive to bronchodilation effects on the cough response.

Treatment with tiotropium bromide mirror the contrasting response observed with salbutamol treatment. Tiotropium bromide did not effect the cough response in normotussive or ozone-sensitised rabbits with a dose of tiotropium bromide sufficient to abolish methacholine induced bronchospasm. In contrast, tiotropium bromide demonstrated a very significant reduction in the ozone sensitised cough responses in guinea pigs at a dose sufficient to abolish methacholine induced bronchospasm. Analysis of the BAL fluid

indicates that tiotropium bromide does not affect the total cell count nor the neutrophilia associated with ozone sensitization in either the rabbits or guinea pigs and this suggests that tiotropium bromide is not acting as an anti-inflammatory. In addition, the dose of tiotropium bromide was enough to abolish methacholine-induced bronchospasm ($250\mu\text{M}$, nebulised for 10 minutes at flow rate of 3 L min^{-1}) in both rabbits and guinea pigs confirming that the dose was sufficiently large (doses were also comparably large to those used by Tashkin *et al.* (2008), Dicipinigaitis *et al.* (2008) and Birrell *et al.* (2014)). In addition to tiotropium bromide, the two experimental anticholinergics CHF-5843 and CHF-6021 from Chiesi Farmaceutici failed to demonstrate any antitussive activity in normotussive or ozone-sensitised rabbits. CHF-6021, like tiotropium bromide, was sufficient to abolish methacholine induced bronchospasm but CHF-5843 was surmountable but by a dose of methacholine 2 log units greater than control. Similarly, BAL indicated that CHF-5843 and CHF-6021 did not reduce the total cell count nor the neutrophilia associated with ozone sensitization. The contrasting response between rabbits and guinea pigs coupled with the similarity of the treatment response to salbutamol treatment and the absence of anti-inflammatory activity indicate that it is probably the bronchodilatory affect of tiotropium bromide that leads to the apparent decrease in the ozone sensitised citric acid induced cough response in guinea pigs. This also suggests that the ozone induced hypertussive response to citric acid in rabbits is demonstrably insensitive to bronchodilation either via activating the sympathetic pathways (β_2 agonism with salbutamol) or antagonising the parasympathetic pathways (Muscarinic antagonism using

tiotropium bromide). This may have a broader implication on the utility of the guinea pig model when validating the pharmacodynamics effect of novel antitussives that have bronchodilatory effects and casts concerns on the assertions that tiotropium bromide is an antitussive based on preclinical experiments based entirely in the guinea pig.

While this study indicates that tiotropium bromide is not acting as an antitussive, it does not explain why the UPLIFT study observed a reduction in the frequency of cough exacerbation (Tashkin *et al.*, 2008). It is possible that tiotropium bromide may assist the mucociliary clearance, and knowing that significant effects on mucociliary clearance can be observed 14 days after treatment in humans (Meyer *et al.*, 2011) and not acutely (Hasani, 2004) then our study design would not have seen observed changes in mucociliary clearance with tiotropium bromide. Thus, it is possible that basis of tiotropium bromide's efficacy in reducing cough exacerbations is tightly bound to the complexity of the aetiology of COPD of which mucociliary clearance may play the largest role in abating cough exacerbations. Alternatively, studies have shown that tiotropium bromide can reduce the cough symptoms of individuals with an acute upper respiratory tract infection (Dicpinigaitis *et al.*, 2008). The mechanism by which pathogens are thought to sensitise the airway to coughing is by acutely damaging the airway epithelia (O'Connell *et al.*, 1996) leading to chronic adaptive changes such as hyperplasia in the mucosa and submucosa (Fujinaka *et al.*, 1985) as well as changes in receptor expression (Kumar *et al.*, 2011) such as TGF β and EGF (Holgate, 2000). However, ozone is also known to cause epithelial damage (Damera *et al.*, 2009; Kosmider *et al.*, 2010) so

tiotropium bromide should be effective if the common cause is non-specific epithelial damage and maladaptation of the epithelia to injury so either the mechanism by which pathogens sensitise the airway is not entirely explained by epithelial damage or the acute exposure to ozone in our model is not sufficient to elicit adaptive changes in the epithelia that lead to hypertussive responses. A recent study demonstrated that tiotropium bromide blocked cough in guinea pigs in a capsaicin induced cough experiment and, further, that this was mediated by TRPV1 receptors specifically (Birrell *et al.*, 2014). We observed a similar effect with the cough response, tiotropium bromide is an effective anti-hypertussive in guinea pigs at a similar dose to Birrell *et al.* (2014), $118\mu\text{g ml}^{-1}$ in our study against $100\mu\text{g ml}^{-1}$ in theirs. A primary issue is that the effect of tiotropium bromide treatment was not mirrored in rabbits, coupled with the observation that tiotropium bromide and salbutamol had similar effectiveness in the guinea pig but not the rabbit our results indicate that you can't separate the primary efficacy of tiotropium bromide (bronchodilation) from its putative secondary effects (TRPV1 modulation) in the guinea pig.

4.6 Conclusion and future work

The relevance of our ozone sensitisation models to clinical disease is that this is a powerful method to study hypertussive cough as opposed to normotussive cough. Patients with intractable cough suffer from a hypersensitivity to cough stimuli, they often cough more frequently and more violently. This thesis objectively demonstrates that ozone sensitisation can be used to make cough

responses more frequent and more reliable between experiments in both rabbits and guinea pigs, enabling the use of cross-over study designs. The utility of these preclinical models may be that they better translate to the disease-state hypertussive cough in man *and* that you can use the same sort of cross-over designs that are popular in clinical trials. There is a dearth of antitussive and anti-hypertussive agents and having a faster, more reliable method to screen compounds *in vivo* can only help speed up that drug development cycle and realizing drugs in man. While we have not elucidated the mechanism of ozone sensitised cough we at the very least have a reliable model, mirrored in two species, that may lead to the discovery of a sensitisation mechanism that explains how hypersensitivity to tussive stimuli occurs which may lead to novel pharmacological targets or approaches.

There may also be a more specific application of the ozone sensitised rabbit and guinea pig models in that ozone sensitisation may be a key environmental route that sensitises idiopathic cough sufferers or exacerbates cough symptoms for COPD, URTI and asthma patients. Ozone levels are affected by the presence of Nitrogen Oxides (NO_x), Volatile Organic Compounds (VOC) and sunlight and the concentrations of NO_x and VOC are typically higher in urban centers (Syri *et al.*, 2001). An increasingly urbanised world population (Drakakis-Smith, 2012) is likely to increase the amount of ozone that people are exposed to and in turn may increase the prevalence of cough exacerbations. The ozone sensitised rabbits and guinea pig models therefore provide the basis to study and model the effect of chronic ozone exposure to the airways as well as test and screen novel compounds.

The guinea pig and rabbit model of ozone sensitised cough were established with positive controls using codeine and levodropropizine and we've gone on to validate and investigate the putative antitussive action of anticholinergic treatments on the cough response. There are number of branches of investigation that could be followed from this thesis.

Firstly, we could further validate the models with a comprehensive battery of putative antitussives in clinical trial such as AP-219, the P2X3 antagonist from Afferent Pharmaceuticals, GSK 2339345, the sodium channel blocker from GlaxoSmithKline and VRP700, from Verona Pharma (see Table 1.2). There is also the possibility of investigating the various OTC cough syrups as we have briefly attempted when we investigated the possible complementary effects of chlorpheniramine and levodropropizine and using a cross-over design to compare common cough syrups to one another. Secondly, the work by Birrell *et al.* (2014) would be interesting to repeat in rabbits because it may reveal that tiotropium bromide can modulate TRPV1 in both the rabbit and the guinea pig. Lastly, the dog would be an interesting species to investigate, partly on the basis of the results from the GSK 2339345 preclinical data (Kwong *et al.*, 2013) and partly because drugs can be administered intrathacally to a conscious animal allowing for smaller doses of agents and probably more reliable delivery of reagents to the lung.

Secondly, repeated and ambient exposure to ozone may induce a chronic cough and is supported by epidemiology of city dwellers exposed to ozone, a by-product of car fumes (Schwartz *et al.*, 1994; Romieu *et al.*, 1997; Groneberg-Kloft *et al.*, 2006; Mao *et al.*, 2013). In our study, we did not observe

any potentiation of ozone sensitisation when we repeatedly exposed the animals to ozone and this is may be because the exposure was not sufficiently prolonged. If ozone can induce a chronic cough then longitudinal studies could test a putative antitussive on acute cough in the first few weeks and then after a period of repeated and ambient exposure could test a putative antitussive on chronic cough. This could be used to investigate novel drugs for chronic cough such as gabapentin (Horton *et al.*, 2008) and thalidomide (Ryan *et al.*, 2012). A cross-over design would be powerful in controlling for individual cough effects as well as being able to *relatively* assess a cough treatments effect on acute and chronic cough. It was observed in this study, that ozone exposure caused animals to cough without citric acid indicating that ozone could be used to sensitise spontaneous cough. A spontaneously coughing animal would be extremely useful for cough research.

Thirdly, the objective measurement of preclinical cough is woefully unmet by the commercial system we tested partly because there is a high rate of false detection and partly because they do not allow the experimenter to playback and listen to audio signal and annotate correction of false positives. However, audio engineering in the music industry has yielded a number of excellent tools to analyze, process and annotate audio files so in the absence of an algorithm to classify a cough sound accurately the workflow of listening and annotating cough sounds manually could be greatly improved by using free software like Sonic visualiser (Sonic Visualiser Copyright © 2005–2012 Chris Cannam and Queen Mary University, London). In addition, by annotating audio signals with where and when a cough has occurred we simultaneously build a supervised

library that a general or machine learning algorithm could use to discover parameters and vectors to base a cough classifier on.

Bibliography

- Abdulqawi, R., R. Dockry, K. Holt, G. Layton, B. G. McCarthy, A. P. Ford, and J. a. Smith (Nov. 2014). "P2X3 receptor antagonist (AF-219) in refractory chronic cough: a randomised, double-blind, placebo-controlled phase 2 study". In: *The Lancet* 6736.14, pp. 1–8. ISSN: 01406736.
- Adcock, J. J., G. J. Douglas, M. Garabette, M. Gascoigne, G. Beatch, M. Walker, and C. P. Page (Feb. 2003). "RSD931, a novel anti-tussive agent acting on airway sensory nerves". In: *British Journal of Pharmacology* 138.3, pp. 407–416. ISSN: 00071188.
- Adcock, J., C. Schneider, and T. Smith (Jan. 1988). "Effects of codeine, morphine and a novel opioid pentapeptide BW443C, on cough, nociception and ventilation in the unanaesthetized guinea-pig". en. In: *British Journal of Pharmacology* 93.1, pp. 93–100. ISSN: 1476-5381.
- Adcock, J. J. (Apr. 2009). "TRPV1 receptors in sensitisation of cough and pain reflexes". In: *Pulmonary Pharmacology & Therapeutics* 22.2, pp. 65–70. ISSN: 1094-5539.
- Adcock, J. J., M. A. Birrell, S. A. Maher, S. J. Bonvini, E. Dubuis, M. A. Wortley, K. Baker, and M. G. Belvisi (2014). "Making Sense Of Sensory Nerves: An In

- Vivo Characterisation Of A δ - And C-Fibres Innervating Guinea-Pig Airways”. In: *C19. New sensations in the lung: the good, the bad, and the nerve*. American Thoracic Society International Conference Abstracts. American Thoracic Society, A3969–A3969.
- Andersson, D. A., C. Gentry, S. Moss, and S. Bevan (Mar. 2008). “Transient Receptor Potential A1 Is a Sensory Receptor for Multiple Products of Oxidative Stress”. en. In: *The Journal of Neuroscience* 28.10, pp. 2485–2494. ISSN: 0270-6474, 1529-2401.
- Armstrong, D., J. Luck, and V. Martin (Feb. 1976). “The effect of emboli upon intrapulmonary receptors in the cat”. In: *Respiration Physiology* 26.1, pp. 41–54. ISSN: 0034-5687.
- Asano, K., Y. Shikama, Y. Shibuya, H. Nakajima, K.-i. Kanai, N. Yamada, and H. Suzaki (Dec. 2008). “Suppressive activity of tiotropium bromide on matrix metalloproteinase production from lung fibroblasts in vitro”. In: *International Journal of Chronic Obstructive Pulmonary Disease* 3.4, pp. 781–790. ISSN: 1176-9106.
- Baekey, D. M., K. F. Morris, S. C. Nuding, L. S. Segers, B. G. Lindsey, and R. Shannon (2003). “Medullary raphe neuron activity is altered during fictive cough in the decerebrate cat”. In: *Journal of Applied Physiology* 94.1, pp. 93–100.
- Baekey, D. M., K. F. Morris, C. Gestreau, Z. Li, B. G. Lindsey, and R. Shannon (July 2001). “Medullary respiratory neurones and control of laryngeal motoneurones during fictive eupnoea and cough in the cat”. en. In: *The Journal of Physiology* 534.2, pp. 565–581. ISSN: 0022-3751, 1469-7793.

- Baker, J. G. (Feb. 2005). "The selectivity of β -adrenoceptor antagonists at the human β_1 , β_2 and β_3 adrenoceptors". en. In: *British Journal of Pharmacology* 144.3, pp. 317–322. ISSN: 1476-5381.
- Barnes, P. J. (Feb. 2000). "The pharmacological properties of tiotropium". In: *CHEST Journal* 117.2_suppl, 63S–66S. ISSN: 0012-3692.
- Bateman, E., S. Rennard, P. Barnes, P. Dicpinigaitis, R. Gosens, N. Gross, J. Nadel, M. Pfeifer, K. Racké, K. Rabe, B. Rubin, T. Welte, and I. Wessler (Dec. 2009). "Alternative mechanisms for tiotropium". In: *Pulmonary Pharmacology & Therapeutics* 22.6, pp. 533–542. ISSN: 1094-5539.
- Belvisi, M. G. (Feb. 2003). "Sensory nerves and airway inflammation: role of A δ and C-fibres". In: *Pulmonary Pharmacology & Therapeutics* 16.1, pp. 1–7. ISSN: 1094-5539.
- Belvisi, M. G. and D. C. Bolser (May 2002). "Summary: Animal Models for Cough". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 249–250. ISSN: 1094-5539.
- Benyamin, R., A. M. Trescot, S. Datta, R. Buenaventura, R. Adlaka, N. Sehgal, S. E. Glaser, and R. Vallejo (Mar. 2008). "Opioid complications and side effects." In: *Pain physician* 11.2 Suppl, S105–20. ISSN: 1533-3159.
- Bergren, D. R. (June 1997). "Sensory receptor activation by mediators of defense reflexes in guinea-pig lungs". In: *Respiration Physiology* 108.3, pp. 195–204. ISSN: 0034-5687.
- Bickerman, H. and A. Barach (Aug. 1954). "The Experimental Production of Cough in Human Subjects Induc... : The American Journal of the Medical Sciences". In: *American Journal of the Medical Sciences* 228.2, pp. 156–163.

- Birrell, M. A., M. G. Belvisi, M. Grace, L. Sadofsky, S. Faruqi, D. J. Hele, S. A. Maher, V. Freund-Michel, and A. H. Morice (Dec. 2009). "TRPA1 Agonists Evoke Coughing in Guinea Pig and Human Volunteers". en. In: *American Journal of Respiratory and Critical Care Medicine* 180.11, pp. 1042–1047. ISSN: 1073-449X, 1535-4970.
- Birrell, M. A., S. J. Bonvini, E. Dubuis, S. A. Maher, M. A. Wortley, M. S. Grace, K. Raemdonck, J. J. Adcock, and M. G. Belvisi (Mar. 2014). "Tiotropium modulates transient receptor potential V1 (TRPV1) in airway sensory nerves: A beneficial off-target effect?" In: *Journal of Allergy and Clinical Immunology* 133.3, 679–687.e9. ISSN: 0091-6749.
- Birring, S., A. Murphy, J. Scullion, C. Brightling, M. Browning, I. Pavord, D. Parker, P. Bradding, and A. Wardlaw (Mar. 2004). "Idiopathic chronic cough and organ-specific autoimmune diseases: a case-control study". In: *Respir.Med.* 98.3, pp. 242–246.
- Birring, S. S. (Mar. 2011). "Controversies in the Evaluation and Management of Chronic Cough". In: *Am. J. Respir. Crit. Care Med.* 183.6, pp. 708–715.
- Bolser, D. C., S. M. Aziz, F. C. DeGennaro, W. Kreutner, R. W. Egan, M. I. Siegel, and R. W. Chapman (Sept. 1993). "Antitussive effects of GABAB agonists in the cat and guinea-pig." In: *British Journal of Pharmacology* 110.1, pp. 491–495. ISSN: 0007-1188.
- Bolser, D. C., F. C. DeGennaro, S. O'Reilly, R. W. Chapman, W. Kreutner, R. W. Egan, and J. A. Hey (Dec. 1994). "Peripheral and central sites of action of GABA-B agonists to inhibit the cough reflex in the cat and guinea pig." In: *British Journal of Pharmacology* 113.4, pp. 1344–1348. ISSN: 0007-1188.

- Bolser, D., F. DeGennaro, S. O'Reilly, J. Hey, and R. Chapman (1995). "Pharmacological studies of allergic cough in the guinea pig". In: *European journal of pharmacology* 277.2-3, pp. 159–164.
- Bolser, D. C. (Feb. 2008). "Older-Generation Antihistamines and Cough Due to Upper Airway Cough Syndrome (UACS): Efficacy and Mechanism". en. In: *Lung* 186.1, pp. 74–77. ISSN: 0341-2040, 1432-1750.
- Bolser, D. C. and P. W. Davenport (May 2002). "Functional Organization of the Central Cough Generation Mechanism". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 221–225. ISSN: 1094-5539.
- (Feb. 2007). "Codeine and cough: an ineffective gold standard". In: *Current opinion in allergy and clinical immunology* 7.1, pp. 32–36. ISSN: 1528-4050.
- Bolser, D. C., F. C. DeGennaro, S. O'Reilly, R. L. McLeod, and J. A. Hey (1997). "Central antitussive activity of the NK1 and NK2 tachykinin receptor antagonists, CP-99,994 and SR 48968, in the guinea-pig and cat". en. In: *British Journal of Pharmacology* 121.2, pp. 165–170. ISSN: 1476-5381.
- Bolser, D. C., J. A. Hey, and R. W. Chapman (Mar. 1999). "Influence of central antitussive drugs on the cough motor pattern". In: *Journal of Applied Physiology* 86.3, pp. 1017–1024.
- Bonham, A. C., S.-i. Sekizawa, C.-Y. Chen, and J. P. Joad (July 2006). "Plasticity of brainstem mechanisms of cough". In: *Respiratory Physiology & Neurobiology* 152.3, pp. 312–319. ISSN: 1569-9048.
- Bonvini, S. J., S. A. Maher, J. J. Adcock, E. Dubuis, M. A. Wortley, J. A. Smith, Anthony P. Ford, Mark A. Birrell, and Maria G. Belvisi (2014). "A Role For ATP And P2X3 In Activation Of Airway Sensory Nerves". In: *American Thoracic*

- Society International Conference Abstracts. American Thoracic Society, A4972–A4972.
- Borison, H. L. (July 1948). “Electrical stimulation of the neural mechanism regulating spasmodic respiratory acts in the cat”. In: *American Journal of Physiology – Legacy Content* 154.1, pp. 55–62.
- Brown, C., W. M. Selig, and J. L. Ellis (Feb. 2007). “Modulation of citric acid-induced cough following lipopolysaccharide-mediated neutrophilia in the guinea pig”. In: *Pulmonary Pharmacology & Therapeutics* 20.1, pp. 90–97. ISSN: 1094-5539.
- Brozmanova, M., L. Mazurova, F. Ru, M. Tatar, and M. Kollarik (Aug. 2012). “Comparison of TRPA1-versus TRPV1-mediated cough in guinea pigs”. In: *European Journal of Pharmacology* 689.1–3, pp. 211–218. ISSN: 0014-2999.
- Caceres, A. I., M. Brackmann, M. D. Elia, B. F. Bessac, D. del Camino, M. D’Amours, J. S. Witek, C. M. Fanger, J. A. Chong, N. J. Hayward, R. J. Homer, L. Cohn, X. Huang, M. M. Moran, and S.-E. Jordt (June 2009). “A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma”. en. In: *Proceedings of the National Academy of Sciences* 106.22, pp. 9099–9104. ISSN: 0027-8424, 1091-6490.
- Cannam, C., C. Landone, and M. Sandler (Oct. 2010). “Sonic Visualiser: An Open Source Application for Viewing, Analysing, and Annotating Music Audio Files”. In: *Proceedings of the ACM Multimedia 2010 International Conference*. Firenze, Italy, pp. 1467–1468.

- Canning, B. J. (Apr. 2009). "Central Regulation of the Cough Reflex: Therapeutic Implications". In: *Pulmonary pharmacology & therapeutics* 22.2, pp. 75–81. ISSN: 1094-5539.
- Canning, B. J., S. B. Mazzone, S. N. Meeker, N. Mori, S. M. Reynolds, and B. J. Udem (June 2004). "Identification of the tracheal and laryngeal afferent neurones mediating cough in anaesthetized guinea-pigs". In: *The Journal of Physiology* 557.2, pp. 543–558. ISSN: 0022-3751.
- Canning, B. J., N. Mori, and S. B. Mazzone (July 2006). "Vagal afferent nerves regulating the cough reflex". In: *Respiratory Physiology & Neurobiology* 152.3, pp. 223–242. ISSN: 1569-9048.
- Canning, B. J. and D. Spina (July 2009). *Sensory Nerves*. Springer, p. 623. ISBN: 9783540790891.
- Carr, M. J., D. D. Hunter, D. B. Jacoby, and B. J. Udem (Apr. 2002). "Expression of Tachykinins in Nonnociceptive Vagal Afferent Neurons during Respiratory Viral Infection in Guinea Pigs". In: *American Journal of Respiratory and Critical Care Medicine* 165.8, pp. 1071–1075. ISSN: 1073-449X.
- Carr, M. J. and B. J. Udem (Sept. 2003). "Bronchopulmonary afferent nerves". en. In: *Respirology* 8.3, pp. 291–301. ISSN: 1440-1843.
- Casaburi, R., D. A. Mahler, P. W. Jones, A. Wanner, G. S. Pedro, R. L. ZuWallack, S. S. Menjoge, C. W. Serby, and T. Witek (Feb. 2002). "A long-term evaluation of once-daily inhaled tiotropium in chronic obstructive pulmonary disease". en. In: *European Respiratory Journal* 19.2, pp. 217–224. ISSN: 0903-1936, 1399-3003.

- Cass, L. J., W. S. Frederik, and J. B. Andosca (Mar. 1954). "Quantitative comparison of dextromethorphan hydrobromide and codeine". eng. In: *The American Journal of the Medical Sciences* 227.3, pp. 291–296. ISSN: 0002-9629.
- Catena, E. and L. Daffonchio (Apr. 1997). "Efficacy and Tolerability of Levodropropizine in Adult Patients with Non-productive Cough. Comparison with Dextromethorphan". In: *Pulmonary Pharmacology & Therapeutics* 10.2, pp. 89–96. ISSN: 1094-5539.
- Chakravarty, N. K., A. Matallana, R. Jensen, and H. L. Borison (Jan. 1956). "Central Effects of Antitussive Drugs on Cough and Respiration". en. In: *Journal of Pharmacology and Experimental Therapeutics* 117.2, pp. 127–135. ISSN: , 1521-0103 (Online).
- Chang, A. B. and P. G. Gibson (May 2002). "Relationship between Cough, Cough Receptor Sensitivity and Asthma in Children". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 287–291. ISSN: 1094-5539.
- Chen, C.-Y., A. C. Bonham, C. G. Plopper, and J. P. Joad (Jan. 2003). "Selected Contribution: Neuroplasticity in nucleus tractus solitarius neurons after episodic ozone exposure in infant primates". en. In: *Journal of Applied Physiology* 94.2, pp. 819–827. ISSN: 8750-7587, 1522-1601.
- Choices, N. (Sept. 2013). *Cough - Treatment*. eng. <http://www.nhs.uk/Conditions/Cough/Pages/Treatment.aspx>. Last Accessed: 2014-08-01.
- Chou, D. T. and S. C. Wang (Jan. 1975). "Studies on the localization of central cough mechanism; site of action of antitussive drugs." en. In: *Journal of*

- Pharmacology and Experimental Therapeutics* 194.3, pp. 499–505. ISSN: , 1521-0103 (Online).
- Chou, Y.-C., J.-F. Liao, W.-Y. Chang, M.-F. Lin, and C.-F. Chen (Mar. 1999). “Binding of dimemorfan to sigma-1 receptor and its anticonvulsant and locomotor effects in mice, compared with dextromethorphan and dextrorphan”. In: *Brain Research* 821.2, pp. 516–519. ISSN: 0006-8993.
- Chung, K. F. and U. G. Lalloo (1996). “Diagnosis and management of chronic persistent dry cough.” In: *Postgraduate medical journal* 72, pp. 594–598. ISSN: 0032-5473.
- Chung, K. F. (Aug. 2003). “Current and future prospects for drugs to suppress cough.” eng. In: *IDrugs : the investigational drugs journal* 6.8, pp. 781–786. ISSN: 1369-7056.
- Chung, K. F. and J. Widdicombe (Nov. 2008). *Pharmacology and Therapeutics of Cough*. Springer, p. 380. ISBN: 9783540798415.
- ClinicalTrials.gov (2014a). *A Study to Assess the Efficacy of AF-219, a P2X3 Receptor Antagonist, in Subjects With Chronic Cough*. <http://clinicaltrials.gov/show/NCT01432730>. [Online. Last Accessed: 2014-08-19 14:23:40].
- (2014b). *The Effect of Pirfenidone on Cough in Patients With Idiopathic Pulmonary Fibrosis*. <http://clinicaltrials.gov/ct2/show/NCT02009293?term=Cough&rank=3> [Online. Last Accessed: 2014-08-19 14:21:00].
- Cohen, H. A., J. Rozen, H. Kristal, Y. Laks, M. Berkovitch, Y. Uziel, E. Kozler, A. Pomeranz, and H. Efrat (Jan. 2012). “Effect of Honey on Nocturnal Cough and

- Sleep Quality: A Double-blind, Randomized, Placebo-Controlled Study”. en. In: *Pediatrics* 130.3, pp. 465–471. ISSN: 0031-4005, 1098-4275.
- Coleridge, J. C. G. and H. M. Coleridge (Jan. 1984). “Afferent vagal C fibre innervation of the lungs and airways and its functional significance”. In: *Reviews of Physiology, Biochemistry and Pharmacology*. Springer Berlin Heidelberg, pp. 1–110. ISBN: 978-3-540-12989-9, 978-3-540-38815-9.
- Cooley, J., J. W. Tukey, and B. J. W. Cooley (1964). “An Algorithm for the Machine Calculation Complex Fourier Series”. In: *Mathematics of Computation* 19.90, pp. 297–301. ISSN: 00255718.
- Coryllos, P. (Oct. 1937). “Action of the Diaphragm in Cough: Experimental and Clinical Study on the Human”. In: *American Journal of the Medical Sciences* 194.4, pp. 523–535.
- Costello, J. F., L. S. Dunlop, and P. J. Gardiner (Oct. 1985). “Characteristics of prostaglandin induced cough in man.” In: *British journal of clinical pharmacology* 20.4, pp. 355–9. ISSN: 0306-5251.
- Cox, I. D., P. J. Wallis, M. C. Apps, D. T. Hughes, D. W. Empey, R. C. Osman, and C. A. Burke (Sept. 1984). “An electromyographic method of objectively assessing cough intensity and use of the method to assess effects of codeine on the dose-response curve to citric acid.” In: *British Journal of Clinical Pharmacology* 18.3, pp. 377–382. ISSN: 0306-5251.
- Crimi, E., A. Spanevello, M. Neri, P. W. Ind, G. A. Rossi, and V. Brusasco (Jan. 1998). “Dissociation between Airway Inflammation and Airway Hyperresponsiveness in Allergic Asthma”. In: *American Journal of Respiratory and Critical Care Medicine* 157.1, pp. 4–9. ISSN: 1073-449X.

- Damera, G., H. Zhao, M. Wang, M. Smith, C. Kirby, W. F. Jester, J. A. Lawson, and R. A. Panettieri (Apr. 2009). "Ozone modulates IL-6 secretion in human airway epithelial and smooth muscle cells". en. In: *American Journal of Physiology - Lung Cellular and Molecular Physiology* 296.4, pp. L674–L683. ISSN: 1040-0605, 1522-1504.
- Davenport, P. W., A. Vovk, R. K. Duke, D. C. Bolser, and E. Robertson (Apr. 2009). "The urge-to-cough and cough motor response modulation by the central effects of nicotine". In: *Pulmonary Pharmacology & Therapeutics* 22.2, pp. 82–89. ISSN: 1094-5539.
- De Blasio, F., P. V. Dicpinigaitis, B. K. Rubin, G. De Danieli, L. Lanata, and A. Zanasi (Jan. 2012). "An observational study on cough in children: epidemiology, impact on quality of sleep and treatment outcome". In: *Cough (London, England)* 8, p. 1. ISSN: 1745-9974.
- Delescluse, I., H. Mace, and J. Adcock (2012). "Inhibition of airway hyper-responsiveness by TRPV1 antagonists (SB-705498 and PF-04065463) in the unanaesthetized, ovalbumin-sensitized guinea pig". en. In: *British Journal of Pharmacology* 166.6, pp. 1822–1832. ISSN: 1476-5381.
- Dicpinigaitis, P. V., A. H. Morice, S. S. Birring, L. McGarvey, J. A. Smith, B. J. Canning, and C. P. Page (Jan. 2014). "Antitussive Drugs—Past, Present, and Future". en. In: *Pharmacological Reviews* 66.2, pp. 468–512. ISSN: , 1521-0081 (Online).
- Dicpinigaitis, P. V. (Mar. 2001). "Effect of the Cyclooxygenase-2 Inhibitor Celecoxib on Bronchial Responsiveness and Cough Reflex Sensitivity in

- Asthmatics". In: *Pulmonary Pharmacology & Therapeutics* 14.2, pp. 93–97. ISSN: 1094-5539.
- Dicpinigaitis, P. V. (2010). "Cough: An Unmet Clinical Need". In: *British Journal of Pharmacology*, no–no. ISSN: 00071188.
- Dicpinigaitis, P., L. Spinner, G. Santhyadka, and A. Negassa (2008). "Effect of Tiotropium on Cough Reflex Sensitivity in Acute Viral Cough". In: *Lung* 186.6, pp. 369–374. ISSN: 0341-2040.
- Doherty, M. J., R. Mister, M. G. Pearson, and P. M. Calverley (2000). "Capsaicin responsiveness and cough in asthma and chronic obstructive pulmonary disease." In: *Thorax* 55, pp. 643–649. ISSN: 0040-6376.
- Doona, M. and D. Walsh (Jan. 1998). "Benzonatate for opioid-resistant cough in advanced cancer". en. In: *Palliative Medicine* 12.1, pp. 55–58. ISSN: 0269-2163, 1477-030X.
- Drakakis-Smith, D. (July 2012). *Urbanisation, Housing and the Development Process*. en. Routledge, p. 260. ISBN: 9781136866180.
- Dubi, A. (1959). "Zur Bedeutung pontiner Atmungssubstrate für den Husten". German. PhD thesis. S.l.
- Duz, M., A. Whittaker, S. Love, T. Parkin, and K. Hughes (Oct. 2010). "Validation of a digital audio recording method for the objective assessment of cough in the horse". In: *Research in Veterinary Science* 89.2, pp. 266–271. ISSN: 0034-5288.
- Dwoskin, L. P. and P. A. Crooks (Jan. 2002). "A novel mechanism of action and potential use for lobeline as a treatment for psychostimulant abuse". In: *Biochemical Pharmacology* 63.2, pp. 89–98. ISSN: 0006-2952.

- Eccles, R., S. Morris, and M. Jawad (June 1992). "Lack of effect of codeine in the treatment of cough associated with acute upper respiratory tract infection". en. In: *Journal of Clinical Pharmacy and Therapeutics* 17.3, pp. 175–180. ISSN: 1365-2710.
- Eccles, R. (July 2006). "Mechanisms of the placebo effect of sweet cough syrups". In: *Respiratory Physiology & Neurobiology. Cough and its Regulation* 152.3, pp. 340–348. ISSN: 1569-9048.
- Eddy, N. B., H. Friebel, K.-J. Hahn, and H. Halbach (1969). "Codeine and its alternates for pain and cough relief". In: *Bulletin of the World Health Organization* 40.3, pp. 425–454. ISSN: 0042-9686.
- Everett, C. F., J. a. Kastelik, R. H. Thompson, and A. H. Morice (Jan. 2007). "Chronic persistent cough in the community: a questionnaire survey." In: *Cough (London, England)* 3, p. 5. ISSN: 1745-9974.
- Fahy, J., H. Wong, P. Geppetti, J. Reis, S. Harris, D. Maclean, J. Nadel, and H. Boushey (1995). "Effect of an NK1 receptor antagonist (CP-99,994) on hypertonic saline- induced bronchoconstriction and cough in male asthmatic subjects – Fahy et al. 152 (3): 879 – American Journal of Respiratory and Critical Care Medicine". In: *Respiratory and Critical Care Medicine* 152.3, pp. 879–884.
- Farrell, M. J., S. Koch, A. Ando, L. J. Cole, G. F. Egan, and S. B. Mazzone (May 2014). "Functionally connected brain regions in the network activated during capsaicin inhalation". en. In: *Human Brain Mapping*, n/a–n/a. ISSN: 1097-0193.

- Featherstone, R. L., P. A. Hutson, S. T. Holgate, and M. K. Church (Jan. 1988). "Active sensitization of guinea-pig airways in vivo enhances in vivo and in vitro responsiveness". en. In: *European Respiratory Journal* 1.9, pp. 839–845. ISSN: 0903-1936, 1399-3003.
- Ferkany, J. W., S. A. Borosky, D. B. Clissold, and M. J. Pontecorvo (June 1988). "Dextromethorphan inhibits NMDA-induced convulsions". In: *European Journal of Pharmacology* 151.1, pp. 151–154. ISSN: 0014-2999.
- Ferrari, S., M. Silva, M. Guarino, J. M. Aerts, and D. Berckmans (Dec. 2008). "Cough sound analysis to identify respiratory infection in pigs". In: *Computers and Electronics in Agriculture* 64.2, pp. 318–325. ISSN: 0168-1699.
- Flockerzi, V. and B. Nilius (Feb. 2007). *Transient Receptor Potential (TRP) Channels*. en. Springer Science & Business Media, p. 611. ISBN: 9783540348917.
- Ford, A. C., D. Forman, P. Moayyedi, and A. H. Morice (Jan. 2006). "Cough in the community: a cross sectional survey and the relationship to gastrointestinal symptoms". en. In: *Thorax* 61.11, pp. 975–979. ISSN: , 1468-3296.
- Fox, A. (Aug. 1995). "Mechanisms and Modulation of Capsaicin Activity on Airway Afferent Nerves". In: *Pulmonary Pharmacology* 8.4–5, pp. 207–215. ISSN: 0952-0600.
- Fox, A., P. Barnes, L. Urban, and A. Dray (1993). "An in vitro study of the properties of single vagal afferents innervating guinea-pig airways." In: *The Journal of Physiology* 469.1, pp. 21–35.

- Fox, A. J., U. G. Laloo, M. G. Belvisi, M. Bernareggi, K. F. Chung, and P. J. Barnes (July 1996). "Bradykinin-evoked sensitization of airway sensory nerves: A mechanism for ACE-inhibitor cough". In: *Nat Med* 2.7, pp. 814–817.
- Freestone, C. and R. Eccles (Oct. 1997). "Assessment of the antitussive efficacy of codeine in cough associated with common cold". In: *J.Pharm.Pharmacol.* 49.10, pp. 1045–1049.
- Fujimura, M., H. Ogawa, M. Yasui, and T. Matsuda (2000). "Eosinophilic tracheobronchitis and airway cough hypersensitivity in chronic non-productive cough". In: *Clinical and Experimental Allergy* 30, pp. 41–47. ISSN: 09547894.
- Fujinaka, L. E., D. M. Hyde, C. G. Plopper, W. S. Tyler, D. L. Dungworth, and L. O. Lollini (Jan. 1985). "Respiratory Bronchiolitis Following Long-Term Ozone Exposure in Bonnet Monkeys: A Morphometric Study". In: *Experimental Lung Research* 8.2-3, pp. 167–190. ISSN: 0190-2148, 1521-0499.
- Gavva, N. R., A. W. Bannon, D. N. Hovland, S. G. Lehto, L. Klionsky, S. Surapaneni, D. C. Immke, C. Henley, L. Arik, A. Bak, J. Davis, N. Ernst, G. Hever, R. Kuang, L. Shi, R. Tamir, J. Wang, W. Wang, G. Zajic, D. Zhu, M. H. Norman, J.-C. Louis, E. Magal, and J. J. S. Treanor (Oct. 2007). "Repeated Administration of Vanilloid Receptor TRPV1 Antagonists Attenuates Hyperthermia Elicited by TRPV1 Blockade". en. In: *Journal of Pharmacology and Experimental Therapeutics* 323.1, pp. 128–137. ISSN: , 1521-0103 (Online).
- Gazzieri, D., M. Trevisani, J. Springer, S. Harrison, G. S. Cottrell, E. Andre, P. Nicoletti, D. Massi, S. Zecchi, D. Nosi, M. Santucci, N. P. Gerard,

- M. Lucattelli, G. Lungarella, A. Fischer, E. F. Grady, N. W. Bunnett, and P. Geppetti (Aug. 2007). "Substance P released by TRPV1-expressing neurons produces reactive oxygen species that mediate ethanol-induced gastric injury". eng. In: *Free Radical Biology & Medicine* 43.4, pp. 581–589. ISSN: 0891-5849.
- Geppetti, P., S. Materazzi, and P. Nicoletti (Mar. 2006). "The transient receptor potential vanilloid 1: Role in airway inflammation and disease". In: *European Journal of Pharmacology. The Pharmacology of the Respiratory Tract* 533.1–3, pp. 207–214. ISSN: 0014-2999.
- Geppetti, P., R. Patacchini, R. Nassini, and S. Materazzi (2010). "Cough: The Emerging Role of the TRPA1 Channel". In: *Lung* 188, pp. 63–68. ISSN: 0341-2040.
- Gibbs-Flournoy, E. A., S. O. Simmons, P. A. Bromberg, T. P. Dick, and J. M. Samet (Mar. 2013). "Monitoring Intracellular Redox Changes in Ozone-Exposed Airway Epithelial Cells". In: *Environmental Health Perspectives* 121.3, pp. 312–317. ISSN: 0091-6765.
- Girard, V., E. Naline, P. Vilain, X. Emonds-Alt, and C. Advenier (Jan. 1995). "Effect of the two tachykinin antagonists, SR 48968 and SR 140333, on cough induced by citric acid in the unanaesthetized guinea pig". en. In: *European Respiratory Journal* 8.7, pp. 1110–1114. ISSN: 0903-1936, 1399-3003.
- Glick, S. D., I. M. Maisonneuve, H. A. Dickinson, and B. A. Kitchen (June 2001). "Comparative effects of dextromethorphan and dextrorphan on morphine, methamphetamine, and nicotine self-administration in rats". In: *European Journal of Pharmacology* 422.1–3, pp. 87–90. ISSN: 0014-2999.

- Gosens, R., D. Rieks, H. Meurs, D. K. Ninaber, K. F. Rabe, J. Nanninga, S. Kolahian, A. J. Halayko, P. S. Hiemstra, and S. Zuyderduyn (Dec. 2009). “Muscarinic M3 receptor stimulation increases cigarette smoke-induced IL-8 secretion by human airway smooth muscle cells”. en. In: *European Respiratory Journal* 34.6, pp. 1436–1443. ISSN: 0903-1936, 1399-3003.
- Government, U. (1986). *Animals (Scientific Procedures) Act 1986*. eng.
- Grattan, T., A. Marshall, K. Higgins, and A. Morice (1995). “The effect of inhaled and oral dextromethorphan on citric acid induced cough in man.” en. In: *British Journal of Clinical Pharmacology* 39.3, pp. 261–263. ISSN: 1365-2125.
- Gravenstein, J. S., R. A. Devloo, and H. K. Beecher (Jan. 1954). “Effect of Antitussive Agents on Experimental and Pathological Cough in Man”. en. In: *Journal of Applied Physiology* 7.2, pp. 119–139. ISSN: 8750-7587, 1522-1601.
- Groneberg-Kloft, B., T. Kraus, A. van Mark, U. Wagner, and A. Fischer (May 2006). “Analysing the causes of chronic cough: relation to diesel exhaust, ozone, nitrogen oxides, sulphur oxides and other environmental factors”. en. In: *Journal of Occupational Medicine and Toxicology* 1.1, p. 6. ISSN: 1745-6673.
- Grootendorst, D. C. and K. F. Rabe (Apr. 2004). “Mechanisms of Bronchial Hyperreactivity in Asthma and Chronic Obstructive Pulmonary Disease”. In: *Proceedings of the American Thoracic Society* 1.2, pp. 77–87. ISSN: 1546-3222.
- GSK (2014). *GSK Clinical Study Register*. [http : / / www . gsk - clinicalstudyregister . com/compounds/gsk2339345#ps](http://www.gsk-clinicalstudyregister.com/compounds/gsk2339345#ps). [Online. Last Accessed: 2014-08-19].

- Gwaltney, J. M., B. Winther, J. T. Patrie, and J. O. Hendley (July 2002). "Combined Antiviral-Antimediator Treatment for the Common Cold". en. In: *Journal of Infectious Diseases* 186.2, pp. 147–154. ISSN: 0022-1899, 1537-6613.
- Haddad, E.-B., H. Patel, J. E. Keeling, M. H. Yacoub, P. J. Barnes, and M. G. Belvisi (May 1999). "Pharmacological characterization of the muscarinic receptor antagonist, glycopyrrolate, in human and guinea-pig airways". en. In: *British Journal of Pharmacology* 127.2, pp. 413–420. ISSN: 1476-5381.
- Hasani, A. (May 2004). "The Effect of Inhaled Tiotropium Bromide on Lung Mucociliary Clearance in Patients With COPD". In: *Chest* 125.5, pp. 1726–1734. ISSN: 0012-3692.
- El-Hashim, A. Z. and S. M. Jaffal (Jan. 2009). "Nerve growth factor enhances cough and airway obstruction via TrkA receptor- and TRPV1-dependent mechanisms". en. In: *Thorax* 64.9, pp. 791–797. ISSN: , 1468-3296.
- Hatzelmann, A., E. J. Morcillo, G. Lungarella, S. Adnot, S. Sanjar, R. Beume, C. Schudt, and H. Tenor (Aug. 2010). "The preclinical pharmacology of roflumilast – A selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease". In: *Pulmonary Pharmacology & Therapeutics* 23.4, pp. 235–256. ISSN: 1094-5539.
- Hay, D. W. P., G. A. M. Giardina, D. E. Griswold, D. C. Underwood, C. J. Kotzer, B. Bush, W. Potts, P. Sandhu, D. Lundberg, J. J. Foley, D. B. Schmidt, L. D. Martin, D. Kilian, J. J. Legos, F. C. Barone, M. A. Luttmann, M. Grugni, L. F. Raveglia, and H. M. Sarau (Jan. 2002). "Nonpeptide Tachykinin Receptor Antagonists. III. SB 235375, a Low Central Nervous System-Penetrant, Potent and Selective Neurokinin-3 Receptor Antagonist, Inhibits Citric Acid-Induced Cough and

- Airways Hyper-reactivity in Guinea Pigs”. en. In: *Journal of Pharmacology and Experimental Therapeutics* 300.1, pp. 314–323. ISSN: , 1521-0103 (Online).
- Heikkinen, T. and A. Järvinen (Jan. 2003). “The common cold”. In: *The Lancet* 361.9351, pp. 51–59. ISSN: 0140-6736.
- Hill, R. (July 2000). “NK1 (substance P) receptor antagonists – why are they not analgesic in humans?” In: *Trends in Pharmacological Sciences* 21.7, pp. 244–246. ISSN: 0165-6147.
- Hj, L., Q. Zm, W. Wl, Y. L, L. Rl, and Z. M (Nov. 2004). “Effects of phosphodiesterase 4 inhibitor on cough response in guinea pigs sensitized and challenged with ovalbumin.” eng. In: *Chinese medical journal* 117.11, pp. 1620–1624. ISSN: 0366-6999.
- Ho, C.-Y., Q. Gu, Y. Lin, and L.-Y. Lee (Sept. 2001). “Sensitivity of vagal afferent endings to chemical irritants in the rat lung”. In: *Respiration Physiology* 127.2–3, pp. 113–124. ISSN: 0034-5687.
- Holgate (2000). “Epithelial damage and response”. en. In: *Clinical & Experimental Allergy* 30, pp. 37–41. ISSN: 1365-2222.
- Horton, M. R., S. K. Danoff, and N. Lechtzin (Jan. 2008). “Thalidomide inhibits the intractable cough of idiopathic pulmonary fibrosis”. en. In: *Thorax* 63.8, pp. 749–749. ISSN: , 1468-3296.
- Hoshiko, K. and J. Morley (Oct. 1993). “Exacerbation of airway hyperreactivity by (+/-)salbutamol in sensitized guinea pig.” eng. In: *Japanese journal of pharmacology* 63.2, pp. 159–163. ISSN: 0021-5198.

- House, A., C. Celly, S. Skeans, J. Lamca, R. W. Egan, J. A. Hey, and R. W. Chapman (May 2004). "Cough reflex in allergic dogs". In: *European Journal of Pharmacology* 492.2-3, pp. 251–258. ISSN: 0014-2999.
- Hwang, S. W., H. Cho, J. Kwak, S.-Y. Lee, C.-J. Kang, J. Jung, S. Cho, K. H. Min, Y.-G. Suh, D. Kim, and U. Oh (May 2000). "Direct activation of capsaicin receptors by products of lipoxygenases: Endogenous capsaicin-like substances". In: *PNAS* 97.11, pp. 6155–6160.
- Ind, P., L. Laitinen, L. Laursen, S. Wenzel, E. Wouters, L. Deamer, and P. Nystrom (Jan. 2003). "Early clinical investigation of Viozan (sibenaedet HCl), a novel D2 dopamine receptor, beta2-adrenoceptor agonist for the treatment of chronic obstructive pulmonary disease symptoms". In: *Respir.Med.* 97 Suppl A, S9–21.
- Irwin, R. S., M. H. Baumann, D. C. Bolser, L.-P. Boulet, S. S. Braman, C. E. Brightling, K. K. Brown, B. J. Canning, A. B. Chang, P. V. Dicpinigaitis, R. Eccles, W. B. Glomb, L. B. Goldstein, L. M. Graham, F. E. Hargreave, P. A. Kvale, S. Z. Lewis, F. D. McCool, D. C. McCrory, U. B. Prakash, M. R. Pratter, M. J. Rosen, E. Schulman, J. J. Shannon, C. Smith Hammond, and S. M. Tarlo (Jan. 2006). "Diagnosis and Management of Cough Executive Summary". In: *Chest* 129.1 suppl, 24S.
- Irwin, R. and M. Madison (Oct. 2010). "The Diagnosis and Treatment of Cough". In:
- Ishiura, Y., M. Fujimura, K. Nobata, M. Abo, T. Oribe, S. Myou, and H. Nakamura (Nov. 2005). "Phosphodiesterase 3 inhibition and cough in elderly asthmatics". en. In: *Cough* 1.1, p. 11. ISSN: 1745-9974.

- Islam, T., R. McConnell, W. J. Gauderman, E. Avol, J. M. Peters, and F. D. Gilliland (Feb. 2008). "Ozone, Oxidant Defense Genes, and Risk of Asthma during Adolescence". In: *American Journal of Respiratory and Critical Care Medicine* 177.4, pp. 388–395. ISSN: 1073-449X.
- Jackson, D. M. (Mar. 1988). "The effect of nedocromil sodium, sodium cromoglycate and codeine phosphate on citric acid-induced cough in dogs." In: *British Journal of Pharmacology* 93.3, pp. 609–612. ISSN: 0007-1188.
- Jackson, D., A. Norris, and R. Eady (1989). "Nedocromil sodium and sensory nerves in the dog lung". In: *Pulmonary Pharmacology* 2.4, pp. 179–184. ISSN: 0952-0600.
- Janson, C., S. Chinn, D. Jarvis, and P. Burney (Jan. 2001). "Determinants of cough in young adults participating in the European Community Respiratory Health Survey". en. In: *European Respiratory Journal* 18.4, pp. 647–654. ISSN: 0903-1936, 1399-3003.
- Jatakanon, A., U. Laloo, S. Lim, K. Chung, and P. Barnes (Mar. 1999). "Increased neutrophils and cytokines, TNF-alpha and IL-8, in induced sputum of non-asthmatic patients with chronic dry cough". In: *Thorax* 54.3, pp. 234–237.
- Jinnai, M., A. Niimi, T. Ueda, H. Matsuoka, M. Takemura, M. Yamaguchi, K. Otsuka, T. Oguma, T. Takeda, I. Ito, H. Matsumoto, and M. Mishima (2010). "Induced sputum concentrations of mucin in patients with asthma and chronic cough." In: *Chest* 137, pp. 1122–1129. ISSN: 1931-3543.
- Joad, J. P., S.-i. Sekizawa, C.-Y. Chen, and A. C. Bonham (Aug. 2007). "Air pollutants and cough". In: *Pulmonary Pharmacology & Therapeutics*. Special

Issue: Fourth International Symposium on Cough 20.4, pp. 347–354. ISSN: 1094-5539.

Joanna Marks-Konczalik, Robert D. Murdoch, Kathryn L. Kelly, Anne Cheesbrough, Sarah K. Siederer, Dave Singh, and Jaclyn A. Smith (May 2014). “Safety, Tolerability, Pharmacokinetics (PK) And Pharmacodynamics (PD) Of GSK2339345 - Novel Sodium Channel Blocker. Results From Two Phase I Studies In Healthy Volunteers”. In: American Thoracic Society International Conference Abstracts. American Thoracic Society, A1607–A1607.

Jones, B. and M. G. Kenward (Mar. 2003). *Design and Analysis of Cross-Over Trials, Second Edition*. en. CRC Press, p. 412. ISBN: 9780412606403.

Kambayashi, T., C. O. Jacob, D. Zhou, N. Mazurek, M. Fong, and G. Strassmann (Nov. 1995). “Cyclic nucleotide phosphodiesterase type IV participates in the regulation of IL-10 and in the subsequent inhibition of TNF-alpha and IL-6 release by endotoxin-stimulated macrophages.” en. In: *The Journal of Immunology* 155.10, pp. 4909–4916. ISSN: 0022-1767, 1550-6606.

Kamei, J. (Oct. 1996). “Role of Opioidergic and Serotonergic Mechanisms in Cough and Antitussives”. In: *Pulmonary Pharmacology* 9.5–6, pp. 349–356. ISSN: 0952-0600.

Kamei, J. (May 2002). “ δ -Opioid Receptor Antagonists as a New Concept for Central Acting Antitussive Drugs”. In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 235–240. ISSN: 1094-5539.

- Kamei, J., H. Tanihara, H. Igarashi, and Y. Kasuya (Sept. 1989). "Effects of N-methyl-D-aspartate antagonists on the cough reflex". In: *European Journal of Pharmacology* 168.2, pp. 153–158. ISSN: 0014-2999.
- Karlsson, J. A. (Oct. 1996). "The Role of Capsaicin-sensitive C-fibre Afferent Nerves in the Cough Reflex". In: *Pulmonary Pharmacology* 9.5–6, pp. 315–321. ISSN: 0952-0600.
- Karlsson, J. A., R. Fuller, Karlsson J.A., and Fuller R.W. (Aug. 1999). "Pharmacological Regulation of the Cough Reflex—from Experimental Models to Antitussive Effects in Man". In: *Pulmonary Pharmacology & Therapeutics* 12.4, pp. 215–228. ISSN: 1094-5539.
- Karlsson, J. A., A. S. Lanner, and C. G. Persson (Jan. 1990). "Airway opioid receptors mediate inhibition of cough and reflex bronchoconstriction in guinea pigs." en. In: *Journal of Pharmacology and Experimental Therapeutics* 252.2, pp. 863–868. ISSN: , 1521-0103 (Online).
- Karlsson, J. A., G. Sant'Ambrogio, and J. Widdicombe (1988). "Afferent neural pathways in cough and reflex bronchoconstriction". In: *Journal of Applied Physiology* 65.3, pp. 1007–1023.
- Khalid, S., R. Murdoch, A. Newlands, K. Smart, A. Kelsall, K. Holt, R. Dockry, A. Woodcock, and J. a. Smith (Mar. 2014). "Transient receptor potential vanilloid 1 (TRPV1) antagonism in patients with refractory chronic cough: A double-blind randomized controlled trial." In: *The Journal of allergy and clinical immunology* 134.1, 56–62.e4. ISSN: 1097-6825.
- Klosterhalfen, B., K. Hörstmann-Jungemann, P. Vogel, S. Flohé, F. Offner, C. J. Kirkpatrick, and P. C. Heinrich (May 1992). "Time course of various

- inflammatory mediators during recurrent endotoxemia”. In: *Biochemical Pharmacology* 43.10, pp. 2103–2109. ISSN: 0006-2952.
- Korpás, J. and G. Kalocsayová (1975). “The expiration reflex in the mouse”. In: *Physiologia Bohemoslovaca* 24.3, pp. 253–256. ISSN: 0369-9463.
- Korpás, J., J. Sadlonová, and M. Vrabec (Oct. 1996). “Analysis of the Cough Sound: an Overview”. In: *Pulmonary Pharmacology* 9.5-6, pp. 261–268. ISSN: 0952-0600.
- Kosmider, B., J. E. Loader, R. C. Murphy, and R. J. Mason (June 2010). “Apoptosis induced by ozone and oxysterols in human alveolar epithelial cells”. In: *Free Radical Biology and Medicine* 48.11, pp. 1513–1524. ISSN: 0891-5849.
- Koto, H., M. Salmon, E.-B. Haddad, T.-J. Huang, J. Zagorski, and K. Fan Chung (July 1997). “Role of Cytokine-induced Neutrophil Chemoattractant (CINC) in Ozone-induced Airway Inflammation and Hyperresponsiveness”. In: *American Journal of Respiratory and Critical Care Medicine* 156.1, pp. 234–239. ISSN: 1073-449X.
- Kotzer, C. J., D. W. P. Hay, G. Dondio, G. Giardina, P. Petrillo, and D. C. Underwood (Jan. 2000). “The Antitussive Activity of δ -Opioid Receptor Stimulation in Guinea Pigs”. en. In: *Journal of Pharmacology and Experimental Therapeutics* 292.2, pp. 803–809. ISSN: , 1521-0103 (Online).
- Kumar, R. K., J. S. Siegle, G. E. Kaiko, C. Herbert, J. E. Mattes, and P. S. Foster (2011). “Responses of Airway Epithelium to Environmental Injury: Role in the Induction Phase of Childhood Asthma”. In: *Journal of Allergy* 2011, pp. 1–7. ISSN: 1687-9783, 1687-9791.

- Kwong, K., Edward F. Webb, Gerald E. Hunsberger, Ruth R. Osborn, Sri Ghatta, Lindsey Ingleby, Michael J. Carr, Jeffrey K. Kerns, and William Rumsey (May 2013). "Pharmacological Characterization of GSK2339345, A Novel Voltage-Gated Sodium Channel Blocker For The Symptomatic Relief Of Cough". In: American Thoracic Society International Conference Abstracts. American Thoracic Society, A4936–A4936.
- Kwong, K. and L.-Y. Lee (Oct. 2002). "PGE2 sensitizes cultured pulmonary vagal sensory neurons to chemical and electrical stimuli". en. In: *Journal of Applied Physiology* 93.4, pp. 1419–1428. ISSN: 8750-7587, 1522-1601.
- Laloo, U. G., A. J. Fox, M. G. Belvisi, K. F. Chung, and P. J. Barnes (Oct. 1995). "Capsazepine inhibits cough induced by capsaicin and citric acid but not by hypertonic saline in guinea pigs". en. In: *Journal of Applied Physiology* 79.4, pp. 1082–1087. ISSN: 8750-7587, 1522-1601.
- Laude, E. A., K. S. Higgins, and A. H. Morice (Sept. 1993). "A comparative study of the effects of citric acid, capsaicin and resiniferatoxin on the cough challenge in guinea-pig and man". In: *Pulmonary pharmacology* 6.3, pp. 171–175. ISSN: 0952-0600.
- Lee, P. C. L., M. S. M. Jawad, and R. Eccles (2000). "Antitussive Efficacy of Dextromethorphan in Cough Associated with Acute Upper Respiratory Tract Infection". en. In: *Journal of Pharmacy and Pharmacology* 52.9, pp. 1137–1142. ISSN: 2042-7158.
- Lee, L.-Y., Q. Gu, and Y.-S. Lin (Jan. 2010). "Effect of smoking on cough reflex sensitivity: basic and preclinical studies." In: *Lung* 188 Suppl, S23–7. ISSN: 1432-1750.

- Lee, L.-Y. and T. E. Pisarri (Mar. 2001). "Afferent properties and reflex functions of bronchopulmonary C-fibers". In: *Respiration Physiology* 125.1–2, pp. 47–65. ISSN: 0034-5687.
- Leung, S. Y., A. Niimi, A. S. Williams, P. Nath, F.-X. Blanc, Q. T. Dinh, and K. F. Chung (Dec. 2007). "Inhibition of citric acid- and capsaicin-induced cough by novel TRPV-1 antagonist, V112220, in guinea-pig". In: *Cough (London, England)* 3, p. 10. ISSN: 1745-9974.
- Lewis, C., C. Ambrose, K. Banner, C. Battram, K. Butler, J. Giddings, J. Mok, J. Nasra, C. Winny, and C. Poll (Aug. 2007). "Animal models of cough: Literature review and presentation of a novel cigarette smoke-enhanced cough model in the guinea-pig". In: *Pulmonary Pharmacology & Therapeutics* 20.4, pp. 325–333. ISSN: 1094-5539.
- Lewis, J. K., B. Bothner, T. J. Smith, and G. Siuzdak (Sept. 1998). "Antiviral agent blocks breathing of the common cold virus". en. In: *Proceedings of the National Academy of Sciences* 95.12, pp. 6774–6778. ISSN: 0027-8424, 1091-6490.
- Lim, K. G., M. A. Rank, P. Y. Hahn, K. A. Keogh, T. I. Morgenthaler, and E. J. Olson (Apr. 2013). "Long-term safety of nebulized lidocaine for adults with difficult-to-control chronic cough: A case series". In: *Chest* 143.4, pp. 1060–1065. ISSN: 0012-3692.
- Liu, Q., M. Fujimura, H. Tachibana, S. Myou, K. Kasahara, and M. Yasui (2001). "Characterization of increased cough sensitivity after antigen challenge in guinea pigs". en. In: *Clinical & Experimental Allergy* 31.3, pp. 474–484. ISSN: 1365-2222.

- LSE (2014). *Phase Iia headline results for VRP700 - London Stock Exchange*.
<http://www.londonstockexchange.com/exchange/news/market-news/market-news-detail.html?announcementId=11991695>. [Online.
Last Accessed: 2014-08-19 13:55:50].
- Lundberg, J. M. and A. Saria (Mar. 1983). “Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants”. en. In: *Nature* 302.5905, pp. 251–253.
- Lunteren, E. van, R. Daniels, E. C. Deal, and M. A. Haxhiu (Jan. 1989). “Role of costal and crural diaphragm and parasternal intercostals during coughing in cats”. In: *J Appl Physiol* 66.1, pp. 135–141.
- Mackenzie, A. J., D. Spina, and C. P. Page (Dec. 2004). “Models used in the development of antitussive drugs”. In: *Drug Discovery Today: Disease Models* 1.3, pp. 297–302. ISSN: 1740-6757.
- Maher, S. A., M. A. Birrell, and M. G. Belvisi (Nov. 2009). “Prostaglandin E2 Mediates Cough via the EP3 Receptor”. In: *American Journal of Respiratory and Critical Care Medicine* 180.10, pp. 923–928. ISSN: 1073-449X.
- Mao, W., W. Xia, and J. Chen (July 2013). “Air pollution and chronic cough in china”. In: *Chest* 144.1, pp. 362–363. ISSN: 0012-3692.
- Mazzone, S. B. *et al.* (2005). “An overview of the sensory receptors regulating cough”. In: *Cough* 1.2, pp. 1–9.
- Mazzone, S. B., L. J. Cole, A. Ando, G. F. Egan, and M. J. Farrell (Feb. 2011). “Investigation of the Neural Control of Cough and Cough Suppression in Humans Using Functional Brain Imaging”. en. In: *The Journal of Neuroscience* 31.8, pp. 2948–2958. ISSN: 0270-6474, 1529-2401.

- Mazzone, S. B., L. McLennan, A. E. McGovern, G. F. Egan, and M. J. Farrell (Aug. 2007). "Representation of Capsaicin-evoked Urge-to-Cough in the Human Brain Using Functional Magnetic Resonance Imaging". en. In: *American Journal of Respiratory and Critical Care Medicine* 176.4, pp. 327–332. ISSN: 1073-449X, 1535-4970.
- Mazzone, S. B., S. M. Reynolds, N. Mori, M. Kollarik, D. G. Farmer, A. C. Myers, and B. J. Canning (Oct. 2009). "Selective Expression of a Sodium Pump Isozyme by Cough Receptors and Evidence for Its Essential Role in Regulating Cough". en. In: *The Journal of Neuroscience* 29.43, pp. 13662–13671. ISSN: 0270-6474, 1529-2401.
- McDonald, J. and D. G. Lambert (Jan. 2005). "Opioid receptors". en. In: *Continuing Education in Anaesthesia, Critical Care & Pain* 5.1, pp. 22–25. ISSN: 1743-1816, 1743-1824.
- McGarvey, L. P. A. and A. J. Ing (Dec. 2004). "Idiopathic cough, prevalence and underlying mechanisms". In: *Pulmonary Pharmacology & Therapeutics*. The 3rd International Symposium on Cough: Acute and Chronic 17.6, pp. 435–439. ISSN: 1094-5539.
- McLeod, R. L., X. Fernandez, C. C. Correll, T. P. Phelps, Y. Jia, X. Wang, and J. A. Hey (2006). "TRPV1 antagonists attenuate antigen-provoked cough in ovalbumin sensitized guinea pigs". In: *Cough* 2.1, p. 10.
- McLeod, R. L., C. C. Correll, Y. Jia, and J. C. Anthes (2008). "TRPV1 antagonists as potential antitussive agents". In: *Lung* 186 Suppl, S59–65. ISSN: 0341-2040.
- Meoni, P., F. C. Tortella, and N. G. Bowery (1997). "An autoradiographic study of dextromethorphan high-affinity binding sites in rat brain:

- sodium-dependency and colocalization with paroxetine”. en. In: *British Journal of Pharmacology* 120.7, pp. 1255–1262. ISSN: 1476-5381.
- Meyer, T., P. Reitmeir, P. Brand, C. Herpich, K. Sommerer, A. Schulze, G. Scheuch, and S. Newman (2011). “Effects of formoterol and tiotropium bromide on mucus clearance in patients with COPD”. In: *Respiratory Medicine* 105, pp. 900–906. ISSN: 09546111.
- Millqvist, E., M. Bende, and O. Löwhagen (1998). *Sensory hyperreactivity—a possible mechanism underlying cough and asthma-like symptoms*. Tech. rep., pp. 1208–1212.
- Millspaugh, C. F. (1892). *American Medicinal Plants: An Illustrated and Descriptive Guide to Plants Indigenous to and Naturalized in the United States which are Used in Medicine*. en. Courier Dover Publications, p. 842. ISBN: 9780486230344.
- Mj, K. (July 1996). “Opiates, opioids and addiction.” eng. In: *Molecular psychiatry* 1.3, pp. 232–254. ISSN: 1359-4184.
- Mohapatra, D. P. and C. Nau (Dec. 2003). “Desensitization of Capsaicin-activated Currents in the Vanilloid Receptor TRPV1 Is Decreased by the Cyclic AMP-dependent Protein Kinase Pathway”. en. In: *Journal of Biological Chemistry* 278.50, pp. 50080–50090. ISSN: 0021-9258, 1083-351X.
- Mokry, J., D. Mokra, G. Nosalova, M. Beharkova, and Z. Feherova (2008). “Influence Of Selective Inhibitors Of Phosphodiesterase 3 And 4 On Cough And Airway Reactivity”. English. In: *Journal Of Physiology And Pharmacology* 59.Suppl 6, pp. 473–482.

- Mokry, J. and G. Nosalova (Nov. 2007). "Evaluation of the cough reflex and airway reactivity in toluene- and ovalbumin-induced airway hyperresponsiveness." In: *Journal of physiology and pharmacology : an official journal of the Polish Physiological Society* 58 Suppl 5.Pt 1, pp. 419–26. ISSN: 0867-5910.
- (Jan. 2011). "The influence of the PDE inhibitors on cough reflex in guinea pigs." In: *Bratislavské lekárske listy* 112.3, pp. 131–5. ISSN: 0006-9248.
- Morice, A. H., G. A. Fontana, M. G. Belvisi, S. S. Birring, K. F. Chung, P. V. Dicpinigaitis, J. A. Kastelik, L. P. McGarvey, J. A. Smith, M. Tatar, and J. Widdicombe (June 2007). "ERS guidelines on the assessment of cough". In: *European Respiratory Journal* 29.6, pp. 1256–1276.
- Morice, A. and R. Abdul-Manap (1998). "Drug treatments for coughs and colds". In: *Prescriber London* 9, pp. 74–78.
- Morice, A. and P. Geppetti (Mar. 2004). "Cough · 5: The type 1 vanilloid receptor: a sensory receptor for cough". In: *Thorax* 59.3, pp. 257–258. ISSN: 0040-6376.
- Morice, A. H. (May 2002). "Epidemiology of Cough". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 253–259. ISSN: 1094-5539.
- (2010). "The cough hypersensitivity syndrome: A novel paradigm for understanding cough". In: *Lung*. Vol. 188. ISBN: 1432-1750 (Electronic).
- Mudge, J. (1779). *A radical and expeditious cure for a recent catarrhus cough: preceded by some observatiours on respiration ; ... To which is added a chapter on the vis vitae, ...* en. E. Allen, p. 288.
- Murao, S., H. Manabe, T. Yamashita, and T. Sekikawa (2008). "Intoxication with Over-the-Counter Antitussive Medication Containing Dihydrocodeine and

- Chlorpheniramine Causes Generalized Convulsion and Mixed Acidosis”. In: *Internal Medicine* 47.11, pp. 1013–1015. ISSN: 0918-2918.
- Muroi, Y., F. Ru, M. Kollarik, B. J. Canning, S. A. Hughes, S. Walsh, M. Sigg, M. J. Carr, and B. J. Udem (Jan. 2011). “Selective silencing of NaV1.7 decreases excitability and conduction in vagal sensory neurons”. en. In: *The Journal of Physiology* 589.23, pp. 5663–5676. ISSN: 0022-3751, 1469-7793.
- Mutolo, D., F. Bongiani, E. Cinelli, G. A. Fontana, and T. Pantaleo (Dec. 2008). “Cough reflex responses during pulmonary C-fibre receptor activation in anesthetized rabbits”. In: *Neuroscience Letters* 448.2, pp. 200–203. ISSN: 0304-3940.
- Mutolo, D., F. Bongiani, G. A. Fontana, and T. Pantaleo (Sept. 2007). “The role of excitatory amino acids and substance P in the mediation of the cough reflex within the nucleus tractus solitarii of the rabbit”. In: *Brain Research Bulletin* 74.4, pp. 284–293. ISSN: 03619230.
- NIH (2014). *How Is Cough Treated?* <http://www.nhlbi.nih.gov/health/health-topics/topics/cough/treatment.html>. Last Accessed: 2014-08-01.
- Niimi, A. (June 2011). “Structural changes in the airways: Cause or effect of chronic cough?” In: *Pulmonary Pharmacology & Therapeutics*. The 2010 Sixth International symposium on Cough; 2010 COUGH 24.3, pp. 328–333. ISSN: 1094-5539.
- Nishitsuji, M., M. Fujimura, Y. Oribe, and S. Nakao (2008). “Effect of montelukast in a guinea pig model of cough variant asthma”. In: *Pulmonary pharmacology & therapeutics* 21.1, pp. 142–145.

- O'Connell, E, V. E. Thomas, J. M. Studham, N. B. Pride, and R. W. Fuller (May 1996). "Capsaicin cough sensitivity increases during upper respiratory infection". In: *Respiratory Medicine* 90.5, pp. 279–286. ISSN: 09546111.
- O'Connell, E (May 2002). "Central Pathways for Cough in Man – Unanswered Questions". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 295–301. ISSN: 1094-5539.
- Oduwole, O., M. M. Meremikwu, A. Oyo-Ita, and E. E. Udoh (June 2014). "Honey for acute cough in children". en. In: *Evidence-Based Child Health: A Cochrane Review Journal* 9.2, pp. 401–444. ISSN: 1557-6272.
- Ohi, Y., H. Yamazaki, R. Takeda, and A. Haji (2005). "Functional and morphological organization of the nucleus tractus solitarius in the fictive cough reflex of guinea pigs". In: *Neuroscience research* 53.2, pp. 201–209.
- Ohi, Y., S. Tsunekawa, and A. Haji (2011). "Dextromethorphan Inhibits the Glutamatergic Synaptic Transmission in the Nucleus Tractus Solitarius of Guinea Pigs". In: *Journal of Pharmacological Sciences* 116.1, pp. 54–62.
- Packman, E. W. and P. E. Ciccone (1983). "The utility of artificially induced cough as a clinical model for evaluating long-lasting antitussive drug combinations". In: *Am J Pharm* 155, pp. 42–48.
- Padma, L. (2013). "Current drugs for the treatment of dry cough." In: *The Journal of the Association of Physicians of India* 61.5 Suppl, pp. 9–13.
- Page, C. P., M. Curtis, M. Walker, and B. Hoffman (2006). *Integrated Pharmacology*. en. Elsevier Mosby, p. 675. ISBN: 9780323040808.
- Page, C. P. and D. Spina (Jan. 2011). "Phosphodiesterase Inhibitors in the Treatment of Inflammatory Diseases". en. In: ed. by S. H. Francis, M. Conti,

- and M. D. Houslay. Handbook of Experimental Pharmacology. Springer Berlin Heidelberg, pp. 391–414. ISBN: 978-3-642-17968-6, 978-3-642-17969-3.
- Patberg, K. W. (June 2011). “The Female Preponderance to Cough Hypersensitivity Syndrome: Another Clue Pointing to the Role of TRPV1 in Cough”. en. In: *Lung* 189.3, pp. 257–258. ISSN: 0341-2040, 1432-1750.
- Paul, I., J. Beiler, McMonagle A, M. Shaffer, Duda L, and Berlin CM (Dec. 2007). “Effect of honey, dextromethorphan, and no treatment on nocturnal cough and sleep quality for coughing children and their parents”. In: *Archives of Pediatrics & Adolescent Medicine* 161.12, pp. 1140–1146. ISSN: 1072-4710.
- Pavesi, L., S. Subburaj, and K. Porter-Shaw (Oct. 2001). “Application and validation of a computerized cough acquisition system for objective monitoring of acute cough*: A meta-analysis”. In: *Chest* 120.4, pp. 1121–1128. ISSN: 0012-3692.
- Piantadosi, S. (May 2013). *Clinical Trials: A Methodologic Perspective*. de. John Wiley & Sons, p. 826. ISBN: 9781118625859.
- Poliacek, I., C. Wang, L. W.-C. Corrie, M. J. Rose, and D. C. Bolser (Apr. 2010). “Microinjection of codeine into the region of the caudal ventral respiratory column suppresses cough in anesthetized cats”. In: *Journal of Applied Physiology* 108.4, pp. 858–865. ISSN: 8750-7587.
- Poulton, T. and J. Francis (1979). “Cough Suppression by Lidocaine.” In: *Anesthesiology* 50.5, pp. 470–472.
- Pounsford, J. C., M. J. Birch, and K. B. Saunders (Sept. 1985). “Effect of bronchodilators on the cough response to inhaled citric acid in normal and asthmatic subjects.” en. In: *Thorax* 40.9, pp. 662–667. ISSN: , 1468-3296.

- Prudon, B., S. S. Birring, D. D. Vara, A. P. Hall, J. P. Thompson, and I. D. Pavord (2005). "Cough and glottic-stop reflex sensitivity in health and disease." In: *Chest* 127, pp. 550–557. ISSN: 0012-3692.
- Ramnarine, S. I., E. B. Haddad, A. M. Khawaja, J. C. Mak, and D. F. Rogers (July 1996). "On muscarinic control of neurogenic mucus secretion in ferret trachea." en. In: *The Journal of Physiology* 494.Pt 2, pp. 577–586. ISSN: 0022-3751, 1469-7793.
- Reynolds, S. M., A. J. Mackenzie, D. Spina, and C. P. Page (Nov. 2004). "The pharmacology of cough". In: *Trends in pharmacological sciences* 25.11, pp. 569–76. ISSN: 0165-6147.
- Rhim, H., S. R. Glaum, and R. J. Miller (Jan. 1993). "Selective opioid agonists modulate afferent transmission in the rat nucleus tractus solitarius." en. In: *Journal of Pharmacology and Experimental Therapeutics* 264.2, pp. 795–800. ISSN: , 1521-0103 (Online).
- Ricciardolo, F. L., V. Rado, L. Fabbri, P. Sterk, G. Di Maria, and P. Geppetti (Feb. 1999). "Bronchoconstriction Induced by Citric Acid Inhalation in Guinea Pigs . Role of Tachykinins, Bradykinin, and Nitric Oxide". In: *Am. J. Respir. Crit. Care Med.* 159.2, pp. 557–562.
- Riccio, M. M., T. Matsumoto, J. J. Adcock, G. J. Douglas, D. Spina, and C. P. Page (1997). "The effect of 15-HPETE on airway responsiveness and pulmonary cell recruitment in rabbits". In: *British Journal of Pharmacology* 122.2, pp. 249–256. ISSN: 00071188.

- Riccio, M., W. Kummer, B. Biglari, A. Myers, and B. Udem (Oct. 1996). "Interganglionic segregation of distinct vagal afferent fibre phenotypes in guinea-pig airways." In: *The Journal of Physiology* 496, pp. 521–530.
- Romieu, I., F. Meneses, S. Ruiz, J. Huerta, J. J. Sienna, M. White, R. Etzel, and M. Hernandez (Sept. 1997). "Effects of Intermittent Ozone Exposure on Peak Expiratory Flow and Respiratory Symptoms among Asthmatic Children in Mexico City". In: *Archives of Environmental Health: An International Journal* 52.5, pp. 368–376. ISSN: 0003-9896.
- Rugg, E. L., D. B. Barnett, and S. R. Nahorski (Jan. 1978). "Coexistence of Beta1 and Beta2 Adrenoceptors in Mammalian Lung: Evidence from Direct Binding Studies". en. In: *Molecular Pharmacology* 14.6, pp. 996–1005. ISSN: , 1521-0111 (Online).
- Ryan, N. M., S. S. Birring, and P. G. Gibson (Nov. 2012). "Gabapentin for refractory chronic cough: a randomised, double-blind, placebo-controlled trial". In: *The Lancet* 380.9853, pp. 1583–1589. ISSN: 0140-6736.
- Sant'Ambrogio, G. (1982). "Information arising from the tracheobronchial tree of mammals". In: *Physiological reviews* 62.2, pp. 531–569.
- Satia, I., K. Holt, H. Badri, M. Woodhead, P. O'Byrne, P. O'Byrne, S. J. Fowler, and J. A. Smith (Jan. 2014). "Neuronal Dysfunction In Asthma; Insights From The Study Of The Cough Reflex". en. In: *Thorax* 69.Suppl 2, A80–A80. ISSN: , 1468-3296.
- Schelegle, E. S., L. J. Gershwin, L. A. Miller, M. V. Fanucchi, L. S. Van Winkle, J. P. Gerriets, W. F. Walby, A. M. Omlor, A. R. Buckpitt, B. K. Tarkington, V. J. Wong, J. P. Joad, K. B. Pinkerton, R. Wu, M. J. Evans, D. M. Hyde, and C. G. Plopper

- (Jan. 2001). "Allergic Asthma Induced in Rhesus Monkeys by House Dust Mite (*Dermatophagoides farinae*)". In: *Am J Pathol* 158.1, pp. 333–341.
- Schroeder, K. and T. Fahey (1996). "Over-the-counter medications for acute cough in children and adults in ambulatory settings". en. In: John Wiley & Sons, Ltd. ISBN: 1465-1858.
- Schroeder, K. and T. Fahey (Feb. 2002). "Systematic review of randomised controlled trials of over the counter cough medicines for acute cough in adults". In: *BMJ* 324.7333, p. 329.
- Schwartz, J., D. W. Dockery, L. M. Neas, D. Wypij, J. H. Ware, J. D. Spengler, P. Koutrakis, F. E. Speizer, and B. G. Ferris (Nov. 1994). "Acute effects of summer air pollution on respiratory symptom reporting in children." In: *American Journal of Respiratory and Critical Care Medicine* 150.5, pp. 1234–1242. ISSN: 1073-449X.
- Sekizawa, K., Y. Xia Jia, T. Ebihara, Y. Hirose, Y. Hirayama, and H. Sasaki (Oct. 1996). "Role of Substance P in Cough". In: *Pulmonary Pharmacology* 9.5-6, pp. 323–328. ISSN: 0952-0600.
- Shams, H., L. Daffonchio, and P. Scheid (1996). "Effects of levodropropizine on vagal afferent C-fibres in the cat". en. In: *British Journal of Pharmacology* 117.5, pp. 853–858. ISSN: 1476-5381.
- Shannon, R., D. M. Baekey, K. F. Morris, and B. G. Lindsey (June 1998). "Ventrolateral medullary respiratory network and a model of cough motor pattern generation". en. In: *Journal of Applied Physiology* 84.6, pp. 2020–2035. ISSN: 8750-7587, 1522-1601.

- Silva, C. R., S. M. Oliveira, M. F. Rossato, G. D. Dalmolin, G. P. Guerra, A. d. S. Prudente, D. A. Cabrini, M. F. Otuki, E. André, and J. Ferreira (June 2011). “The involvement of TRPA1 channel activation in the inflammatory response evoked by topical application of cinnamaldehyde to mice”. In: *Life Sciences* 88.25–26, pp. 1077–1087. ISSN: 0024-3205.
- Simera, M., M. Veternik, and I. Poljacek (Jan. 2013). “Naloxone Blocks Suppression of Cough by Codeine in Anesthetized Rabbits”. en. In: ed. by M. Pokorski. *Advances in Experimental Medicine and Biology*. Springer Netherlands, pp. 65–71. ISBN: 978-94-007-4548-3, 978-94-007-4549-0.
- Smith, J. and A. Woodcock (Sept. 2006). “Cough and its importance in COPD”. In: *International Journal of Chronic Obstructive Pulmonary Disease* 1.3, pp. 305–314. ISSN: 1176-9106.
- Smith, S. W. (2003). *Digital Signal Processing: A Practical Guide for Engineers and Scientists*. en. Elsevier, p. 666. ISBN: 9780750674447.
- Stone, R. A., Y. M. Worsdell, R. W. Fuller, and P. J. Barnes (Jan. 1993). “Effects of 5-hydroxytryptamine and 5-hydroxytryptophan infusion on the human cough reflex”. en. In: *Journal of Applied Physiology* 74.1, pp. 396–401. ISSN: 8750-7587, 1522-1601.
- Suzuki, T., Y. Masukawa, and M. Misawa (Dec. 1990). “Drug interactions in the reinforcing effects of over-the-counter cough syrups”. en. In: *Psychopharmacology* 102.4, pp. 438–442. ISSN: 0033-3158, 1432-2072.
- Syri, S., M. Amann, W. Schöpp, and C. Heyes (June 2001). “Estimating long-term population exposure to ozone in urban areas of Europe”. In: *Environmental Pollution* 113.1, pp. 59–69. ISSN: 0269-7491.

- Takahama, K. and T. Shirasaki (July 2007). "Central and peripheral mechanisms of narcotic antitussives: codeine-sensitive and -resistant coughs". en. In: *Cough* 3.1, p. 8. ISSN: 1745-9974.
- Tanaka, M. and K. Maruyama (Sept. 2005). "Mechanisms of capsaicin- and citric-acid-induced cough reflexes in guinea pigs". In: *Journal of Pharmacological Sciences* 99.1, pp. 77–82. ISSN: 1347-8613.
- Tashkin, D. P., B. Celli, S. Senn, D. Burkhart, S. Kesten, S. Menjoge, and M. Decramer (2008). "A 4-Year Trial of Tiotropium in Chronic Obstructive Pulmonary Disease". In: *New England Journal of Medicine* 359.15, pp. 1543–1554. ISSN: 0028-4793.
- Tatar, M., S. E. Webber, and J. G. Widdicombe (1988). "Lung C-fibre receptor activation and defensive reflexes in anaesthetized cats." In: *The Journal of physiology* 402.1, pp. 411–420.
- Tatar, M., D. Karcolova, R. Pecova, and M. Brozmanova (Oct. 1996). "The Role of Partial Laryngeal Denervation on the Cough Reflex in Awake Guinea-pigs, Rats and Rabbits". In: *Pulmonary Pharmacology* 9.5–6, pp. 371–372. ISSN: 0952-0600.
- Taylor-Clark, T. E. and B. J. Undem (2010). "Ozone activates airway nerves via the selective stimulation of TRPA1 ion channels". en. In: *The Journal of Physiology* 588.3, pp. 423–433. ISSN: 1469-7793.
- Tenenbaum, A., E. Grossman, J. Shemesh, E. Z. Fisman, I. Nosrati, and M. Motro (Jan. 2000). "Intermediate but not low doses of aspirin can suppress angiotensin-converting enzyme inhibitor-induced cough". en. In: *American Journal of Hypertension* 13.7, pp. 776–782. ISSN: 0895-7061, 1941-7225.

- Tortella, F. C., M. Pellicano, and N. G. Bowery (Dec. 1989). "Dextromethorphan and neuromodulation: old drug coughs up new activities". In: *Trends in Pharmacological Sciences* 10.12, pp. 501–507. ISSN: 0165-6147.
- Trevisani, M., A. Milan, R. Gatti, A. Zanasi, S. Harrison, G. Fontana, A. H. Morice, and P. Geppetti (2004). "Antitussive activity of iodo-resiniferatoxin in guinea pigs". In: *Thorax* 59.9, pp. 769–772.
- Trevisani, M., J. Siemens, S. Materazzi, D. M. Bautista, R. Nassini, B. Campi, N. Imamachi, E. Andrè, R. Patacchini, G. S. Cottrell, R. Gatti, A. I. Basbaum, N. W. Bunnett, D. Julius, and P. Geppetti (Aug. 2007). "4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1". en. In: *Proceedings of the National Academy of Sciences* 104.33, pp. 13519–13524. ISSN: 0027-8424, 1091-6490.
- Udem, B. J. (2004). "Subtypes of vagal afferent C-fibres in guinea-pig lungs". In: *The Journal of Physiology* 556.3, pp. 905–917. ISSN: 0022-3751.
- Udem, B. J. and M. J. Carr (May 2001). "Pharmacology of airway afferent nerve activity". en. In: *Respiratory Research* 2.4, p. 234. ISSN: 1465-9921.
- Udem, B. J., M. J. Carr, and M. Kollarik (May 2002). "Physiology and Plasticity of Putative Cough Fibres in the Guinea Pig". In: *Pulmonary Pharmacology & Therapeutics* 15.3, pp. 193–198. ISSN: 1094-5539.
- Watanabe, N., S. Horie, G. J. Michael, S. Keir, D. Spina, C. P. Page, and J. V. Priestley (2006). "Immunohistochemical co-localization of transient receptor potential vanilloid (TRPV)1 and sensory neuropeptides in the guinea-pig respiratory system". In: *Neuroscience* 141.3, pp. 1533–1543. ISSN: 0306-4522.

- Wellek, S. and M. Blettner (Apr. 2012). "On the Proper Use of the Crossover Design in Clinical Trials". In: *Deutsches Ärzteblatt International* 109.15, pp. 276–281. ISSN: 1866-0452.
- WHO (2008). "Chronic Respiratory Diseases". In: *Aids Research Programme* 1, pp. 001–156.
- (2012). "Dextromethorphan Pre-Review Report". In: June, pp. 4–8.
- Widdicombe, J. (Oct. 1996a). "Sensory Mechanisms". In: *Pulmonary Pharmacology* 9.5–6, pp. 383–387. ISSN: 0952-0600.
- Widdicombe, J. (1954). "Respiratory reflexes excited by inflation of the lungs". In: *The Journal of Physiology* 123.1, pp. 105–115.
- (July 1995). "Neurophysiology of the cough reflex". In: *European Respiratory Journal* 8.7, pp. 1193–1202.
- (Nov. 1996b). "Sensory neurophysiology of the cough reflex". In: *Journal of Allergy and Clinical Immunology* 98.5, Part 2, S84–S90. ISSN: 0091-6749.
- (Mar. 2001). "Airway receptors". In: *Respiration Physiology* 125.1-2, pp. 3–15. ISSN: 0034-5687.
- Widdicombe, J., M. Tatar, G. Fontana, J. Hanacek, P. Davenport, F. Lavorini, and D. Bolser (June 2011). "Workshop: Tuning the 'cough center'". In: *Pulmonary Pharmacology & Therapeutics* 24.3, pp. 344–352. ISSN: 1094-5539.
- Wise, P. M., P. A. S. Breslin, and P. Dalton (Feb. 2014). "Effect of Taste Sensation on Cough Reflex Sensitivity". en. In: *Lung* 192.1, pp. 9–13. ISSN: 0341-2040, 1432-1750.

- Wollin, L. and M. Pieper (Aug. 2010). "Tiotropium bromide exerts anti-inflammatory activity in a cigarette smoke mouse model of COPD". In: *Pulmonary Pharmacology & Therapeutics* 23.4, pp. 345–354. ISSN: 1094-5539.
- Wong, G. Y. and N. R. Gavva (Apr. 2009). "Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks". In: *Brain Research Reviews* 60.1, pp. 267–277. ISSN: 0165-0173.
- Woo-Jung Song, Yoon-Seok Chang, Kyung-Hwan Lim, Shoab Faruqi, Jana Plevkova, Suk-Il Chang, Sang-Heon Cho, and Alyn H. Morice (May 2014). "Epidemiology Of Chronic Cough In General Adult Populations: A Systematic Review And Meta-Analysis". In: American Thoracic Society International Conference Abstracts. American Thoracic Society, A6587–A6587.
- Woolf, C. R. and a. Rosenberg (Mar. 1964). "Objective Assessment of Cough Suppressants Under Clinical Conditions Using a Tape Recorder System." In: *Thorax* 19, pp. 125–30. ISSN: 0040-6376.
- Xiang, A., Y. Uchida, A. Nomura, H. Iijima, F. Dong, M.-J. Zhang, and S. Hasegawa (Nov. 1998). "Effects of airway inflammation on cough response in the guinea pig". In: *Journal of Applied Physiology* 85.5, pp. 1847–1854.
- Xiu, Q., M. Fujimura, M. Nomura, M. Saito, T. Matsuda, N. Akao, K. Kondo, and K. Matsushima (Jan. 1995). "Bronchial hyperresponsiveness and airway neutrophil accumulation induced by interleukin-8 and the effect of the thromboxane A2 antagonist S-1452 in guinea-pigs". en. In: *Clinical & Experimental Allergy* 25.1, pp. 51–59. ISSN: 1365-2222.

Young, E. C. and J. A. Smith (June 2011). “Pharmacologic therapy for cough”. In: *Current Opinion in Pharmacology* 11.3, pp. 224–230. ISSN: 1471-4892.

Zhang, W., L. Fievez, E. Cheu, F. Bureau, W. Rong, F. Zhang, Y. Zhang, C. Advenier, and P. Gustin (Feb. 2010). “Anti-inflammatory effects of formoterol and ipratropium bromide against acute cadmium-induced pulmonary inflammation in rats”. In: *European Journal of Pharmacology* 628.1–3, pp. 171–178. ISSN: 0014-2999.