Nanoparticles, sensory irritation and the lung: Environmental influences on biological processes.

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Thesis abstract

Diesel exhaust particles (DEP) have been shown in epidemiological studies to be associated with respiratory symptoms. The cellular mechanisms driving these effects continue to be investigated. However, one aspect of lung physiology that has not been studied is the potential for DEP to activate transient receptor potential (TRP) ion channels expressed on airway sensory nerves. The aim of this thesis was to determine whether DEP could activate airway sensory nerves and the underlying mechanisms involved.

In the anesthetised guinea pig, it was established that DEP induced action potential firing in chemosensitive C-fibre airway afferents *in vivo*, but had no effect on mechanosensitive A-δ fibres. DEP was also shown to activate airway sensory nerves *in vitro*. Physicochemical characterisation of DEP indicated that it was mainly composed of inorganic carbon with the remainder organic hydrocarbons and trace impurities. It was demonstrated that the organic components of DEP (DEP-OE) were capable of activating the vagus nerve, while the carbon core had no effect.

With the use of pharmacological tools and tissue from genetically modified mice, activation of sensory nerves by DEP-OE *in vitro* was found to be mediated by TRPA1. DEP-OE induced action potential firing was also inhibited by TRPA1 antagonism *in vivo*. Application of antioxidants abolished responses to DEP-OE, demonstrating a role for oxidative stress which is a known activator of TRPA1. Polycyclic aromatic hydrocarbons (PAHs) present in DEP-OE were found to activate sensory nerves and this activation was also inhibited by TRPA1 antagonism. PAHs responses were inhibited by aryl hydrocarbon receptor (AhR) antagonists. Responses to DEP-OE were also inhibited by AhR antagonists, and were reduced in $AhR^{-/-}$ mice. It was hypothesised that AhR induced oxidative stress through an association with the mitochondria, and the application of a specific mitochondrial superoxide scavenger inhibited responses to DEP-OE.

A novel neuronal mechanism by which DEP exposure can activate airway sensory nerves has been identified. Potential therapeutic targets may be developed to protect susceptible individuals from the adverse health effects of air pollution. In a wider context, the results of this thesis could also be used as additional evidence for the need to reduce the levels of diesel emissions in the urban environment.

Statement of Originality

I declare that the following work presented in this thesis is entirely my own work, except where specific reference is made.

In vivo single fibre recordings were carried out with the assistance of Dr. John Adcock, as these experiments required a Home Office Licence certification relating to the use of neuromuscular blockers. Certain physicochemical analytical techniques were carried out with the assistance of Dr. Shu Chen (TGA and EDX), Dr. Catriona McGilvery (cryo-EM) and Dr. Sheng Hu (Soxhlet extraction).

Research was performed at the National Heart and Lung Institute, the Department of Chemistry and Department of Materials Science at Imperial College London, London, UK.

This work has not been previously submitted for the reward of a degree course at this or any other university.

Ryan Robinson

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List of Abbreviations

4αPDD	4α-phorbol 12,13-didecanoate
ACE	Angiotensin-Converting-Enzyme
AHNT	Aryl Hydrocarbon Nuclear Translocator
AhR	Aryl Hydrocarbon Receptor
AITC	Allyl-isothiocyanate
AP-1	Activator Protein 1
ATP	Adenosine Triphosphate
BAL	Bronchial Alveolar Lavage
BEAS-2B	Human bronchial epithelial cell line BEAS-2B
bp	Base Pairs
CB	Carbon Black
cDNA	complementary DNA
COPD	Chronic Obstructive Pulmonary Disorder
COX-2	Cyclooxygenase-2
cryo-EM	Cryogenic Electron Microscopy
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DCM	Dichloromethane
DEP	Diesel Exhaust Particles
DEP-OE	Organic Extract of DEP (SRM-1975)
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DLS	Dynamic Light Scattering
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DPF	Diesel Particulate Filters
DRG	Dorsal Root Ganglia
ECS	Extracellular Solution
EPA	Environmental Protection Agency
FEV_1	Forced expiratory volume in 1 second
FVC	Forced Vital Capacity
GCPR	G-coupled Protein Receptors
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSH	Reduced Glutathione
GSK101	GSK1016790a
GSK219	GSK2193874
IFN-g	Interferon Gamma
IgE	Immunoglobulin E
IIAM	International Institute for the Advancement of Medicine
IL-4	Interlukin-4
IL-6	Interlukin-6
IL-8	Interlukin-8
Jan 130	Janssen 130
K_{50}	50 mmol/L Potassium chloride solution
KO	Knock Out
LMWA	Low Molecular Weight Antioxidants
LPS	Lipopolysaccharides
MMP-1	Matrix metalloproteinase-1

mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin-resistant Staphylococcus aureus
NAC	N-acetylcysteine
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIST	National Institute of Standards and Technology
NTS	Nucleus Tractus Solitarious
org-DEP	Organic Extract of DEP (self made)
OVA	Ovalbumin
PAH	Polycyclic Aromatic Hydrocarbons
par-DEP	cleaned Particles of DEP (self made)
PAR_2	Protease Activated Receptor 2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PGE_2	Prostaglandin E2
PM	Particulate Matter
$PM_{0.1}$	Particulate Matter less than 0.1 μm in aerodynamic diameter.
PM_{10}	Particulate Matter less than 10 μm in aerodynamic diameter.
$PM_{2.5}$	Particulate Matter less than 2.5 μm in aerodynamic diameter.
RARs	Rapidly Adapting Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Real Time PCR
RTX	Resinoferotoxin
SARs	Slowly adapting receptors
SEM	Standard Error of the Mean
TBE	Tris/Borate/EDTA
TCDD	2,3,7,8-tetrachloro[1,6-[3H]]dibenzo-p-dioxin
TEM	Transmission Electron Microscopy
TEM-EDX	Transmission Electron Microscopy Energy Dispersive X-ray
TGA	Thermogravimetric Analysis
TMF	6,2',4'-trimethoxyflavone
TRPs	Transient Receptor Potential Ion Channels
TTX	Tetrodotoxin
VGKCs	Voltage Gated Potassium channels
VGSCs	Voltage Gated Sodium channels
WHO	World Health Organisation
WT	Wild Type
Xention	Xention D0501

1 Chapter One: Introduction

1.1 Urban air pollution

The world continues to experience rapid industrialisation and population growth, with the percentage of the global population residing in urban areas increasing from 35.6% in 1965 to 54% in 2015 (United Nations, Department of Economic and Social Affairs, Population Division - World Urbanization Prospects: The 2014 Revision, custom data acquired via website). Such urbanisation has given rise to 'megacities', defined as cities with populations in excess of ten million. The total number of megacities is expected to increase to 27 by 2025, up from just 3 in 1975 (UN - World Urbanization Prospects: The 2014 Revision). Explosive expansion is driven by migration from rural areas to more prosperous urban areas (Taubenböck *et al.*, 2012). However, such expansion is accompanied by a number of issues including the ability to provide the necessary levels of infrastructure, crime prevention and sanitisation (Wenzel *et al.*, 2007). Another common problem that all megacities face is poor air quality (Molina & Molina, 2004). With densely populated areas containing automobiles and industrial facilities, an increasing number of individuals are now being exposed to urban air pollution and there is growing concern about its effects on public health (Molina *et al.*, 2004; Parrish & Zhu, 2009; Gurjar *et al.*, 2010). The World Health Organization (WHO) has identified urban air pollution as the world's leading environmental health risk, with exposure associated with increased morbidity and mortality (Cesaroni *et al.*, 2013; Fischer *et al.*, 2015).

Urban air pollution is highly heterogeneous in its composition (Sun *et al.*, 2004; Boogaard *et al.*, 2011). Air pollutants are a complex mixture of gases and particulate matter (PM), which are small particles suspended in the air (Kelly & Fussell, 2011). Although air pollutants can be derived from natural sources such as volcanoes or dust storms, in an urban environment the overwhelming majority of such pollutants originate from man-made combustion sources (Delmelle *et al.*, 2002; Kwon *et al.*, 2002; Longo *et al.*, 2010). The exact composition of air pollution at any one location is highly dependent on temporal and spatial factors, as well as the point source of origin (Chow *et al.*, 1994; Perry & Gee, 1995; Kim Oanh *et al.*, 2006).

With such variability, one of the main challenges in the past few decades has been to identify the precise components and mechanisms responsible for the adverse health effects of air pollution. While substantial progress has been made, the complex relationships between sources, composition and health effects ensure that further investigation is required. A greater

understanding of the risks of exposure to anthropogenic air pollution may allow for the development of less harmful fuel types, treatments to protect the public or simply provide a solid evidence base to enable policy changes that will legislate for meaningful improvements in air quality.

1.1.1 A brief history of air pollution

Co-ordinated efforts to understand the hazards of air pollution began around the middle of the 20th century. However, human beings have been releasing combustion products into the air since our ancestors first discovered and harnessed fire. In Israel, burnt seeds and wood were identified at an archaeological site indicating that humans were in control of fire around 790,000 years ago (Goren-Inbar *et al.*, 2004) and wood combustion continued to be a primary form of energy production up until relatively recently in recorded history (Ausset *et al.*, 1998). Many parts of the world still rely on biomass as a fuel source to heat homes and to cook with (Smith *et al.*, 2004; Northcross *et al.*, 2010).

For major cities, the composition of air pollution changed dramatically with the Industrial Revolution that occurred in the latter half of the 18th century. This period ushered in a new era of technological advancement around the world, driven by the invention of the combustion engine and mass production manufacturing methodology (Cannadine, 1984). Unfortunately, these advancements came at an environmental price. Significant quantities of fossil fuels, such as coal, were utilised as a source of energy to power the machinery of the Industrial Revolution and major quantities of pollutants were released into the atmosphere as a result (Flick, 1980). Factories and processing facilities often were built in close proximity to each other, creating localised areas with extremely high level of air pollution. However, air pollutants were not just restricted to their immediate geographical vicinity of their origin and could be carried thousands of kilometres. Geological surveys have revealed that heavy metals can be found in ice deposits in Greenland that correlated with the start of the Industrial Revolution, despite the area not having a significant industrial presence at the time (Candelone *et al.*, 1995). Similar results were found in samples taken from sediments of Swedish lakes (Bränvall *et al.*, 2001). During this period few were concerned about the potential health effects of air pollution and the majority of households were heated with coal fires that often released emissions directly into the living areas (Rudge, 2012). A commonly held thought at the time was that exposure to air pollution was in fact beneficial to an individual, acting as a sort of airborne disinfectant to protect against other diseases such as cholera (Thorsheim, 2006).

While there is some evidence that some individuals were beginning to be concerned (Anderson, 2009), it was not until a few key incidents during the 20th century that there was a united effort to understand the risks of air pollution. One such event occurred in the Meuse Valley region of Belgium. The area was one of the most heavily industrialised in continental Europe and was affected as an extremely dense fog between the 1st and 5th of December, 1930 (Nemery *et al.*, 2001). Within a few days, hundreds of residents experienced significant respiratory symptoms and over sixty deaths were reported. This event was so severe that it made headlines across the world, both in scientific journals and public newspapers. An expert committee was formed to investigate the cause of the morbidity and mortality observed. It was noted that the first signs of symptoms coincided with the 3rd day of the fog, and symptoms rapidly alleviated after the fog dissipated (Firket, 1936). Post mortem investigations revealed that toxicological results were negative, although abnormalities were observed in the lung and airways. It was concluded that the symptoms and deaths observed were from airway irritation due to contact with the ambient air. This extreme air pollution event occurred due to an unfortunate combination of regional geography and uncommon metrological conditions. The residents of the Meuse Valley were surrounded by hills up to 120m high on either side and in a geographical corridor with the industrialised cities of Liege and Huy at either end. During this period, air was trapped at ground level and pollutants precipitated out in cold and virtually motionless ambient air as fog.

While the Meuse Valley event was one of the first air pollution events to gain global attention, the London Fog of 1952 has gained a reputation as being one of the deadliest. Another significant fog manifested between the 5th and the 8th of December (Davis, 2002), due to an anticyclone arriving from the north-west and trapping cold, stationary air over the city (Brimblecombe, 2006). Pollutants were unable to dissipate and very quickly a dramatic increase in deaths was noted. The weekly death rates prior to the London Fog averaged around 800-900 per week before spiking to nearly 2,500 in the post-Fog week (Scott, 1953). In the immediate aftermath, it was clear that the deaths coincided with significant increases in the recorded levels of air pollution (Fig 1.1). Death rates continued to stay elevated for weeks afterwards. A retrospective analysis estimated that around 12,000 additional deaths occurred as a result of the fog (Bell & Davis, 2001). The London Fog has been attributed as the catalyst for the signing of the Clean Air Act (1956) following a government led inquest to understand the threat air pollution presented to society (Brimblecombe, 2006). As well as enabling legal change, the London Fog was also a major driver for the scientific community to begin to thoroughly investigate the health effects of air pollution.

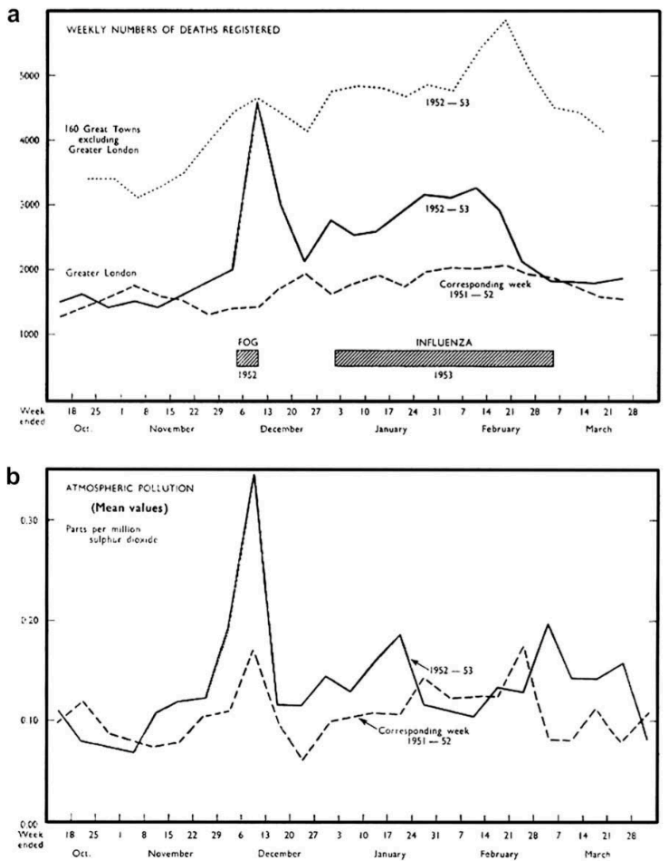


Figure 1.1: Records of weekly deaths and atmospheric pollution in London between October 1952 and March 1953.

(a) *Weekly deaths recorded in Greater London compared to other major towns, and with the corresponding period in 1951– 1952.* ***(b)*** *Mean values of sulphur dioxide compared with 1951–1952. Copyright: H R Anderson (2009)*

In the decades that have followed, numerous studies have now identified associations between exposure to air pollution and a range of adverse health effects, generally affecting the cardiovascular system and the respiratory system as well as links with cancer (Dockery & Pope, 1994; Atkinson *et al.*, 2001; Brunekreef & Holgate, 2002; Dominici *et al.*, 2006; Kelly & Fussell, 2015; Samoli *et al.*, 2016). While the majority of individuals do not severely suffer overtly upon exposure to air pollution (Fig 1.2), epidemiological analysis has revealed a variety of ways in which air pollutants may cause harm especially in susceptible individuals.

Analysis of hospital admissions has demonstrated that exposure to air pollution is associated with cardiac disease, such as ischaemic heart disease (Le Tertre *et al.*, 2002); as well as myocardial infections and pneumonia (Zanobetti & Schwartz, 2006) Similar associations were found between air pollution exposure and hospital admissions for heart disease in the elderly. (Schwartz, 1999). In a large analysis across 12 million Medicare enrolees in the U.S, an

increased risk of emergency hospitalisation for cardiovascular and respiratory admissions was observed during air pollution events (Peng *et al.*, 2009). Increased hospital admissions for asthma and chronic obstructive pulmonary disorder (COPD) related issues are also associated with air pollution (Atkinson *et al.*, 2001). Results from cohort studies also confirm that long term exposure to air pollution is associated with morbidity and mortality in a similar manner, as well as links with lung cancer (Abbey *et al.*, 1999; Fischer *et al.*, 2015). Using predicted values for average exposure levels in Europe, the proportion of lung cancer due to air pollution is estimated to be as high as 10.7% (Boffetta, 2006). However, there is at present limited evidence for associations between air pollution and other forms of cancer (Boffetta & Nyberg, 2003).

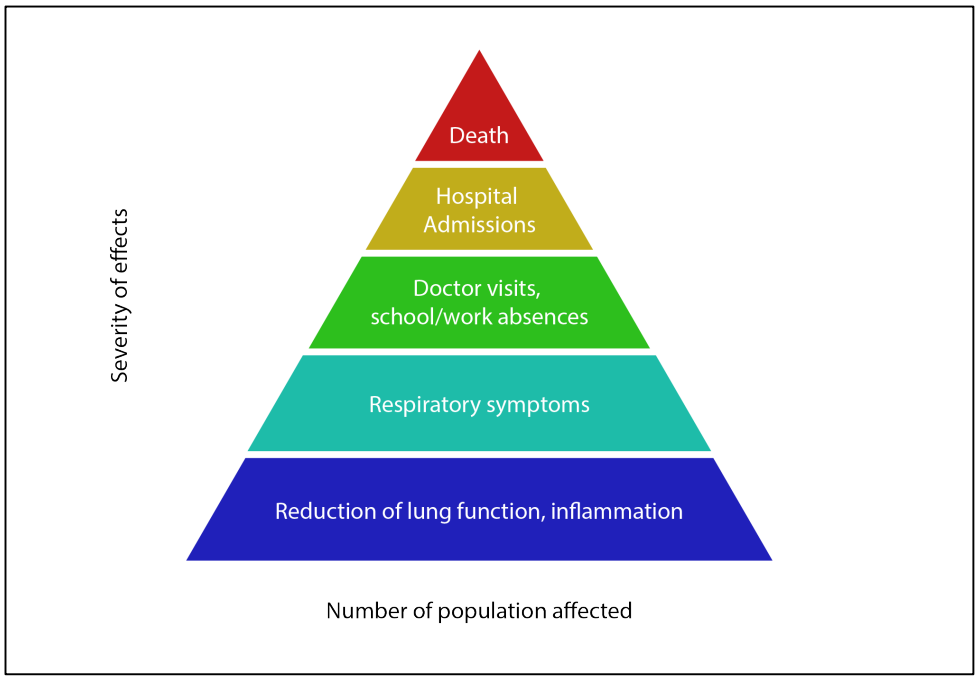


Figure 1.2: Air pollution health effects pyramid.
Air pollution affects a large proportion of the population, however many only are affected in a subtle and sub-clinical manner. But depending on the sensitivity of the individual, the effects of air pollution can become increasingly severe although the number of individuals affected in such a manner decreases.

1.1.2 Air pollutant classification

Air pollutants are a complex mixture with variable physicochemical composition. Even within the same city, samples of ambient air at different times and locations can contain very different pollutants, due to differences in point sources, metrological conditions and other

environmental factors. (Chow *et al.*, 1994; Perry & Gee, 1995; Kim Oanh *et al.*, 2006) Such variation makes drawing firm conclusions from epidemiological data difficult, as it is almost impossible know the specific pollution exposure profile for an individual. However, air pollution can be broadly separated into two main categories: gaseous components and PM (Alexis *et al.*, 2004). Additionally, air pollutants can be classified as either primary pollutants that are generated directly from a point source or secondary pollutants that are formed in the environment from chemical reactions of primary pollutants (Na *et al.*, 2004; Volkamer *et al.*, 2006).

Gaseous components of air pollution include carbon monoxide (CO), nitrogen oxides (NO_x), sulphur oxides (SO_x) and ozone (O_3). CO is produced from the incomplete combustion of fossil fuels and can transiently build up to relatively high levels in tunnels and parking garages (Raub *et al.*, 2000). An odourless and tasteless gas, CO is extremely dangerous it binds irreversibly to haemoglobin and prevents normal oxygen transport in the blood stream. CO is the cause of a significant number of accidental deaths and poisonings every year (Homer *et al.*, 2005). CO exposure in pregnant women has also been linked to low birth weights (B Ritz, 1999). NO_x is a major urban pollutant and is an important marker for traffic emissions (Ban-Weiss *et al.*, 2008). NO_x exposure induces a range of respiratory symptoms including cough, nasal congestion and exacerbations in asthma (Chen *et al.*, 2007). SO_x is another pollutant that is formed during the combustion of sulphur containing fossil fuels such as coal (Spörl *et al.*, 2013). SO_x exposure induces dyspnoea and bronchoconstriction in asthmatic patients (Balmes *et al.*, 1987) O_3 is a secondary pollutant formed from the rapid oxidation of other pollutants such as CO in the presence of NO_x, and can be transported across large geographical distances to influence global pollutant burdens (Fiore *et al.*, 2002). O_3 exposure is associated with acute myocardial infarctions (Ruidavets *et al.*, 2005) and chronic low level O_3 exposure is associated with respiratory symptoms such as wheeze and difficulty breathing in new born infants, particularly for those with asthmatic mothers (Triche *et al.*, 2006).

While the health effects of gaseous pollutants are significant, an increasing focus is being placed upon the effects of PM which can remain in the atmosphere for an extended duration, and be translocated far from its point source (Schwarze *et al.*, 2006; Sacks *et al.*, 2011). Modern scientific interest in the effects of PM can be traced back to epidemiological studies in the early 1990s that identified unexpected health effects from relatively low concentrations of PM (Pope & Dockery, 2006). Many of the adverse health effects of air pollution exposure have been correlated to PM concentration, rather than other aspects of air pollution (Zanobetti & Schwartz, 2006). Only PM remains significantly associated with out of hospital myocardial infarctions when adjustments for other pollutants were made. (Kang *et al.*, 2016). Unlike

gaseous pollutants, it is difficult to measure and experimentally test the effects of the highly heterogenic PM that are present in real world (Schwarze *et al.*, 2006).

1.2 Airborne particulate matter

1.2.1 Composition and sources of PM

Although PM samples from different locations will vary in their composition (Davidson *et al.*, 2005), the major components typically include sulphates, nitrates, ammonium, chloride, soil, dust, elemental carbon and organic hydrocarbons (Harrison & Yin, 2000; Sun *et al.*, 2004). PM can also contain a wide range of metals species such as sodium, magnesium, calcium, titanium, iron, zinc, lead, manganese and copper, although typically these form a small fraction of the total PM mass (Bell *et al.*, 2009; Jeng, 2010). Iron, chromium and copper are particularly elevated around street level locations (Boogaard *et al.*, 2011).

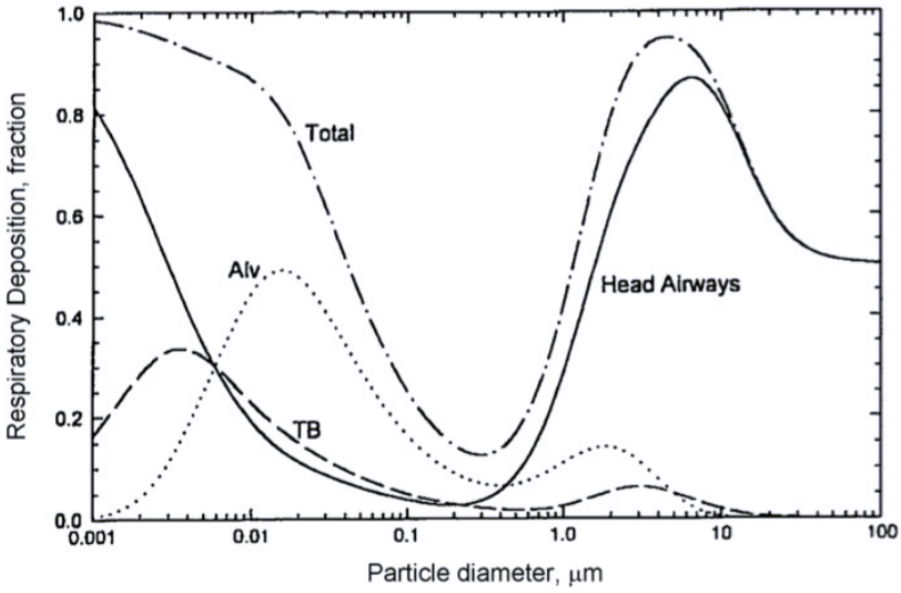


Figure 1.3: Deposition of particles in the airways.

Average predicted total and regional deposition of particles for nose breathing individuals. Alv: alveolar region; TB: tracheobronchial region. Copyright: (*Aerosol Technology*, 2012)

Of all the characteristics, aerodynamic diameter is one of the most commonly used properties used to differentiate and classify PM. This size classification is based on the influence that aerodynamic diameter has on the deposition potential of PM within the lungs and airways (Carvalho *et al.*, 2011) (Fig 1.3). 'Coarse' particulates are known as PM_{10}, which are particles

between 10μm and 2.5μm in aerodynamic diameter. PM_{10} can be composed of resuspensions of dust and soil, as well as bioaerosols including pollen, mould and spores (Perez *et al.*, 2008). PM_{10} are capable of staying airborne and depositing into the larger airways when inhaled. Owing to technical limitations of the past, previous studies focused mainly on PM_{10} when looking at the effects of air pollution. However, in more recent times focus has turned towards 'fine particulates' which are primarily generated from man-made sources (Magliano, 1999). Known as $PM_{2.5}$, these particulates have an aerodynamic diameter less than 2.5μm and can be deposited into the lower regions of the lung, including smaller airways and alveoli (Kampa & Castanas, 2008). Continuing this trend, the most recent studies have identified 'ultrafine particulates' as $PM_{0.1}$, which are less than 100nm in diameter (Oberdörster *et al.*, 2005). These ultrafine particles appear to exhibit enhanced toxicity for a given mass, likely due to their greater surface area and ability to be inhaled deep into the lungs but also to pass through the alveolar barrier, translocate systemically and penetrate cell membranes (Nel, 2006). Materials that are commonly thought to be biologically inert have been shown be harmful when inhaled as ultrafine particles (Tetley, 2007).

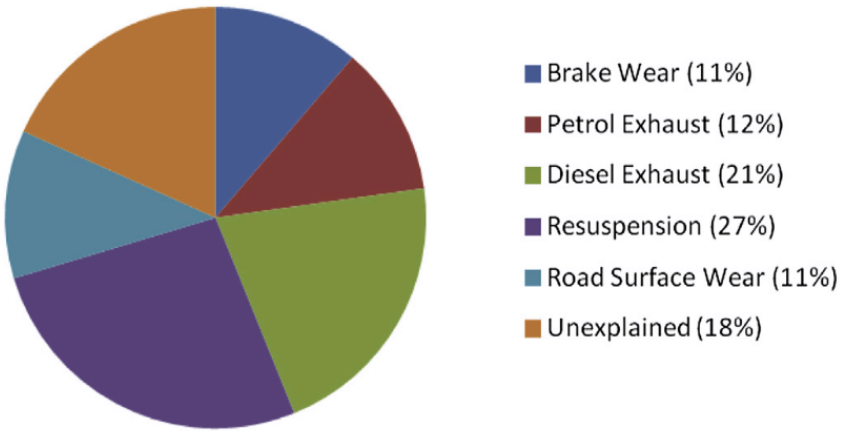


Figure 1.4: Source appointment to $PM_{2.5}$.

Source contribution for $PM_{2.5}$ as sampled within a tunnel. Source: (Lawrence *et al.*, 2013)*.*

The point source of PM is another potential method of classification. In an urban environment, anthropogenic sources dominate PM burden. Motor vehicles heavily contribute to PM levels in an urban environment (Fig 1.4), and specifically the exhaust emissions from diesel engines contribute to a significant proportion of urban PM (Fraser *et al.*, 1999). Manufacturing and industrial processes also contribute to the PM burden (Moreno *et al.*, 2004; Querol *et al.*, 2004). Other non-exhaust derived PM can include dust ablated from road surfaces as well as break wear, tyre wear and road surface abrasion (Thorpe & Harrison, 2008). It is also worth noting that there are natural sources of PM, although their relative contributions are highly

variable between different geographical areas (Delmelle *et al.*, 2002; Sandström & Forsberg, 2008). For example, windswept dust from the Sahara-Sahel desert in Africa is regularly deposited in southern Europe (Perez *et al.*, 2008).

1.2.2 Risks of PM exposure

As the lung and airways are in intimate contact with the environment, it is not surprising that PM has been found to have effects on the respiratory system. Within 8 European cities, the concentration of PM and black smoke were found to be positively associated with emergency hospital admissions for patients with underling respiratory illnesses such as asthma and COPD (Atkinson *et al.*, 2001). In a large cohort study, increases in mortality were associated with sulphate and fine particulate air pollution in U.S. cities (Dockery & Pope, 1994) Lung function can also be adversely affected by particulate matter, such as reductions in forced expiratory function over one second (FEV_1) in children (Hoek & Brunekreef, 1994; Dassen *et al.*, 2012). PM is also thought to affect the cardiovascular system, with associations found between cardiac dysfunction, thrombosis, hypertension and atherosclerosis (Nelin *et al.*, 2012). Elevated levels of $PM_{2.5}$ within vehicles are associated with alterations in heart rate variability (Riediker *et al.*, 2004).

A consistent finding within the literature is that there are sub-groups within the population who appear to be more susceptible to the effects of PM (Sacks *et al.*, 2011). For individuals with underlying health conditions, high levels of PM can often exacerbate underlying symptoms. An association between PM and allergic respiratory diseases has been demonstrated across several studies (Dávila *et al.*, 2007). Exposure to PM is associated with exacerbations in asthmatics, with increased hospital visits due to asthmatic symptoms following a day of higher levels of PM_{10} (Schwartz *et al.*, 1993; Gavett & Koren, 2001). COPD is a disease characterized by a progressive and non-reversible airflow limitation (Ito & Barnes, 2009). Exacerbations of respiratory symptoms can be life threatening in COPD patients, and there are clear links between increased levels of PM and mortality in those with COPD (Sunyer *et al.*, 2000; Zanobetti *et al.*, 2008).

Age can also influence susceptibility to PM, even in the absence of underlying health complications. During the winter months children have an increased risk of respiratory illness associated due to PM exposure, as well as SO_2 and NO_x (Mutius *et al.*, 1995). Post neonates showed the highest relative risk of mortality against daily PM_{10} levels compared to other age groups (Ha *et al.*, 2003). There is also evidence to suggest that exposure to PM can lead to

abnormal long term development of the lung, with a study of Southern Californian school children demonstrating that over an 8 year period exposure to PM results in reductions in forced vital capacity (FVC) (Gauderman *et al.*, 2004). The elderly represents another population group that are more susceptible to the effects of PM. High levels of $PM_{2.5}$ are associated with lower autonomic control in elderly individuals (Liao *et al.*, 1999). Increased exposure to traffic derived PM is also associated with an accelerated decline of FVC and FEV_1 in the elderly (Lepeule *et al.*, 2014).

It is unsurprising that individuals with multiple risk factors appear to be acutely sensitive to the effects of PM. In children with underlying respiratory symptoms, episodes of cough are associated with exposure to PM (Tiittanen *et al.*, 1999; Ostro *et al.*, 2001). Increased respiratory symptoms, along with a reduction of peak expiratory flow by more than 10%, were associated with increased PM in children with existing bronchial hyper-responsiveness and higher immunoglobulin E (IgE) serum levels, whilst in other groups of children no associations could be found (Boezen *et al.*, 1999). Similarly, children with a history of wheeze or asthma had stronger associations with respiratory symptoms and PM concentration than those that did not (Dockery *et al.*, 1989). PM_{10} also raises the rates of paediatric asthma exacerbations and hospital admissions even after a lag period of 1-4 days (Nastos *et al.*, 2010).

1.2.3 Identifying the critical properties of PM.

With exposure to PM being clearly associated with a range of adverse health effects, attempts have been made to identify the precise components of PM and the related mechanisms that are responsible. Epidemiological studies face disadvantages due to the diverse composition of PM, the varying exposure profiles of the population and additional factors such as underlying health conditions or additive exposure to other irritants. Despite these challenges, the size of PM has been highlighted as playing an important role in determining toxicity (Schwarze *et al.*, 2006). Several studies now suggest that $PM_{2.5}$ as being a hazardous fraction of PM (Vanvliet *et al.*, 1997; Kunzli *et al.*, 2000; Nicolai *et al.*, 2003; Codispoti *et al.*, 2015). It is estimated that $PM_{2.5}$ is responsible for approximately 0.8 million premature deaths per year (Cohen *et al.*, 2005). However, it should be noted that other studies have not found any positive associations with $PM_{2.5}$ and health effects, highlighting the fact that particle size alone may not be the only defining property of PM that drives adverse health effects (Strak *et al.*, 2012; Cox & Popken, 2015) It is likely that PM composition also plays a role. In a study comparing naturally derived PM with PM that originates from man-made sources, only the latter was found to be associated with mortality (Pope *et al.*, 1999; Schwartz *et al.*, 1999).

$PM_{2.5}$ does contain a significant proportion of carbonaceous particles, and elemental carbon has the greatest percentage increase in risk of hospital admissions compared to other components of PM (Bell *et al.*, 2009).

Whether smaller particles are more toxic due to their inherent particle size or whether it is due to a contrasting chemical composition is still not certain. However, the small carbonaceous particles present in $PM_{2.5}$ are generated from the combustion of fossil fuels and therefore it is unsurprising motor vehicles are a major source of such $PM_{2.5}$. Although petrol cars remain popular, in recent decades the prevelance of diesel cars has dramatically increased and now constitutes 50% of the vehicle fleet in Europe (Cames & Helmers, 2013) (Fig 1.5). In such regions, the emissions from diesel engines are responsible for the majority of $PM_{2.5}$ in urban environments, with an analysis of PM comparing a rural sample site with a sample site next to a German autobahn finding three times the number of diesel particles next to the roadside (Vogt *et al.*, 2003). It is for this reason that the PM emitted from diesel engines has been studied extensively, and is the focus of this thesis.

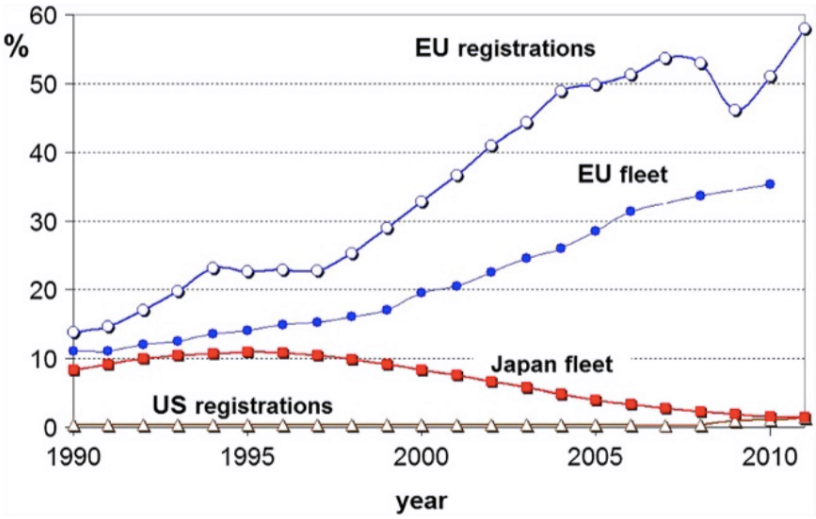


Figure 1.5: Diesel vehicle market penetration across the world.

Data showing the increasing popularity of diesel vehicles has primarily been restricted to the European markets. Data is expressed either as percentage of new car registrations or total fleet number. Copyright: Cames et al. (2013)

1.3 Diesel Exhaust Particles

It is now well accepted that diesel exhaust particles (DEP) form a significant contribution to the total PM burden in urban areas. In a report issued by the Department of Environment (UK

1995), across the whole of the country DEP contributed to 24% of total PM mass. However in London, an major urban environment, DEP was found to contribute to 87% of total PM mass, highlighting the important differences in PM composition in cities. Nanoparticle sized (5 nm to 50 nm) DEPs contributed to only 1-20% of total diesel particulate mass, but they contributed to over 90% of DEPs as counted by particle number (Kittelson, 1998). Given their prevalence and the strong associations between urban PM and adverse health effects, the effects of diesel emissions have been a major focus for air pollution research (Sydbom *et al.*, 2001; Kagawa, 2002).

1.3.1 Diesel engine technology

Advancements in technologies such as electrical batteries and hydrogen fuels have led to the development of low emission personal transportation vehicles (Bakker *et al.*, 2012), however the vast majority of vehicles on the road are powered by traditional internal combustion engines. The combustion engine is a centuries old technology, with the first combustion engine being built in 1876 by Nikolaus Otto. The fundamental principal of the internal combustion engine is that fuel is introduced into the engine and combusted in a controlled manner. The fuel combustion generates high temperatures and pressures to drive pistons that produce torque, which in turn can be used as effective power. The majority of motor vehicles incorporate combustion engines that run on either petrol or diesel fuels, with some key differences between each in terms of engine technology. Petrol engines function by using a spark ignition system, while diesel engines function by using a compression ignition system. In a diesel engine, air is introduced into the engine cylinder and is compressed by the action of the piston, dramatically increasing its temperature as pressure increases. Diesel fuel is then sprayed into the cylinder, and instantly ignited due to the compressed air's extreme temperature. The resulting explosion pushes the piston back and generates useful power, while exhaust gases and particulates are vented out of the cylinder and the process is repeated. Diesel fuel itself differs in composition from petrol. Although they are both based from petroleum oil, diesel is a less refined fraction than petrol and contains heavier hydrocarbons.

Diesel engines are capable of generating greater power and torque than petrol engine. This is because the compression of the fuel/air mixture ensures that the diesel fuel, which is inherently more energy dense than petrol, is burnt more efficiently. Additionally, the cost of fuel for diesel has traditionally been lower than petrol on a price per litre comparison, although recent times have brought parity between the two fuel types. Due to these advantages diesel

engines are the engines of choice for heavier duty transport vehicles, locomotives, maritime vehicles and industrial machinery, as well as an increasing number of privately owned motor vehicles (Lloyd & Cackette, 2001).

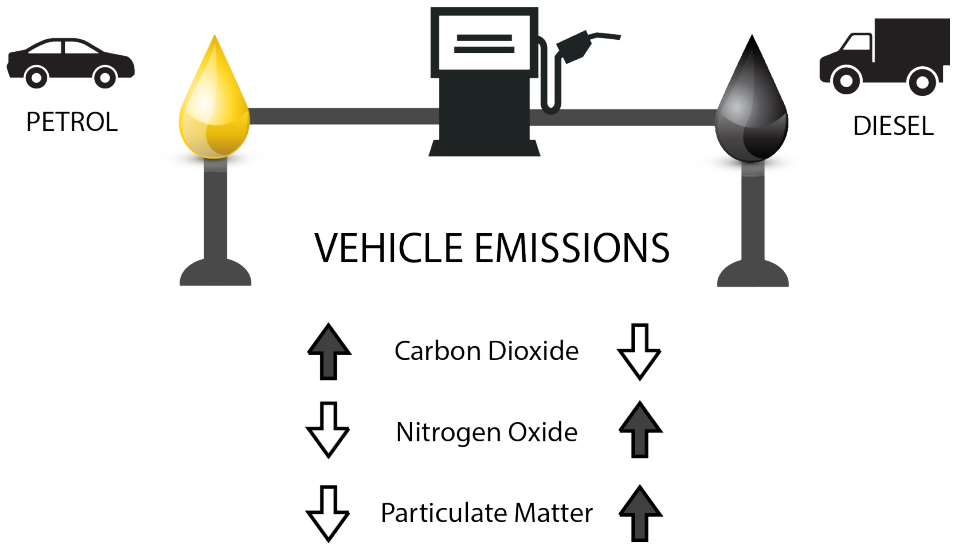


Figure 1.6: Comparison of emissions from petrol and diesel powered vehicles.
Due to differences in the fuel composition and combustion techniques, the emissions from petrol based vehicles emit greater amounts of carbon dioxide, while diesel based vehicles emit greater amounts of nitrogen oxides and particulate matter.

While compression theoretically improves the efficiency of fuel combustion, in reality diesel engines emit a range of emissions due to incomplete combustion of fuel. Diesel emissions are mainly composed of NO_x and DEP, as well as some SO_x and CO. When comparing the emissions of petrol and diesel vehicles, petrol engines produce comparatively greater levels of CO_2 while diesel engines produce larger amounts of NO_X (Geller *et al.*, 2006) (Figure 1.6). NO_X emissions are closely regulated, as secondary reactions with NO_X, organic compounds and sunlight results in the production of hazardous O_3. Analysis of the particulates gathered from the engines of various motor vehicles also demonstrates that diesel engines produce significantly more PM than petrol engines, which explains why diesel vehicles dominate as a source of urban PM (Cheung *et al.*, 2009). Such differences in tail pipe emissions can be attributed to chemistry of the original fuels used, and the manner in which the fuel in combusted.

Following concern with regard to diesel emissions and subsequent regulation, a number of technologies are now found within diesel engines that are able to reduce their emissions.

These include fuel injectors, physical filters, gaseous active and passive regeneration systems (Walker, 2004; Geller *et al.*, 2006; Khalek *et al.*, 2015). For example, urea can be used as a catalytic reduction agent to reduce NO_x emissions from diesel engines. Innovative diesel engine technologies are being explored to improve the efficiency of the combustion process that will also reduce emissions (Knecht, 2008). Alterations to fuel composition, such as the incorporation of soy-based biodiesel to fuel mixtures, have been demonstrated to alter the emissions produced (Ratcliff *et al.*, 2010). In a study investigating the efficacy of particulate filters on the air that flows within a cabin, particulate filters were capable of reducing the concentration of DEP by 50%, but importantly, there was no significant change in intensity of symptoms reported unless activated carbon filters were utilised (Rudell *et al.*, 1999). The most common use of such diesel particulate filters (DPF) are to control the tail pipe emissions of DEP and can be demonstrated to reduce a significant proportion of emitted DEP mass. While this technology is relatively mature, it is important to note that while DPFs can successfully remove many of the larger diameter DEP, there is a tendency for these filters to increase nanoparticle sized DEP emissions (Vaaraslahti *et al.*, 2004). Therefore, the continued presence of nanoparticle sized DEP in the exhaust emissions of diesel vehicles remains a cause for concern particularly, as airborne nanoparticles have been implicated as a hazardous component of air pollution (Morawska *et al.*, 2008).

1.3.2 Physicochemical composition of DEP

The general composition of DEP consists of a carbon core surrounded by absorbed organic hydrocarbons and embedded trace impurities such as transition metals, sulphates and nitrates (BeruBe *et al.*, 1999; Sharma *et al.*, 2005; Wichmann, 2007) (Figure 1.7). The precise characteristics of any given sample of DEP are variable, as differences in the operating conditions of a diesel engine can influence the size of DEP (Lidia Morawska *et al.*, 1998; Wong *et al.*, 2003; Shah *et al.*, 2004; Sharma *et al.*, 2005). Differences in fuel composition, such as low sulphur and low aromatic diesel fuels, can also significantly change the organic content of emitted DEP (Ålander *et al.*, 2004). Secondary modifications by atmospheric factors such as ozone and ultra-violet radiation can also alter the properties and toxicity of DEP (Kafoury & Kelley, 2005; Li *et al.*, 2009a).

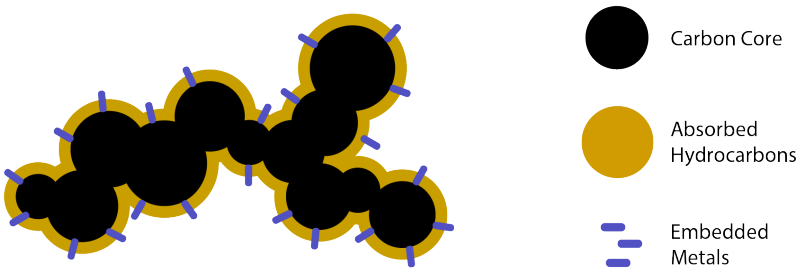


Figure 1.7: Composition of DEP.

DEP are composed of carbon cores surrounded by absorbed hydrocarbons, embedded trace metals and other impurities. Primary particles aggregate to form larger chain like structures.

1.3.2.1 Carbon core

The carbon core of DEP forms the bulk of the particulate mass. With regards to particle size, primary DEP (the particles that are immediately generated during the combustion process) have been shown to be non-spherical in shape and can range from 20-50nm in aerodynamic diameter (Murphy *et al.*, 1999; Shi *et al.*, 2000; Kocbach *et al.*, 2005). Analysis of size distributions have shown that around 60% of particles can be classified as ultrafine when considering total particle number (BeruBe *et al.*, 1999). DEP are formed initially by a process of nucleation, whereby precursor molecules grow into small carbon core nuclei (Neeft *et al.*, 1996). However, after the initial generation of primary DEP, larger agglomerates readily form fractal chain structures that can be up to several microns in diameter (Braun *et al.*, 2004; Chen *et al.*, 2005; Fujitani *et al.*, 2009). Differences in fuel types can also promote the formation of agglomeration due to the presence of impurities such as sulphur or other additives (Park *et al.*, 2003)

1.3.2.2 Organic components

A diverse range of embedded organic compounds are found surrounding the carbon core of DEP, namely polycyclic aromatic hydrocarbons (PAHs) and their derivatives (R Zimmermann *et al.*, 2000; Poster *et al.*, 2003; 2009; Callén *et al.*, 2013). PAHs are well established as environmental pollutants, with both mutagenic and carcinogenic properties (Pelkonen & Nebert, 1982; Samanta *et al.*, 2002; Srogi, 2007; Kim *et al.*, 2013). One third of the total PAH emissions released in the USA are attributable to motor vehicles (Ramdahl *et al.*, 1983) and contributions from diesel vehicles contribute to about 2/3 of total PAH emissions (Nielsen *et*

al., 1996). Sources of PAHs in motor vehicle emissions can include unburnt fuel, lubricating oil and pyrosynthesis from low weight hydrocarbons (Marr *et al.*, 1999). Unlike gasoline based fuels, the original PAH content in diesel fuels is not always well correlated with the concentrations of PAHs emitted, potentially indicating that the importance of non-fuel sources in diesel emissions (Marr *et al.*, 1999). However, when comparing pure diesel fuels with biodiesel blends up to 20% (v/v) the addition of biodiesel resulted in increases in certain specific PAH (Corrêa & Arbilla, 2006).

PAHs are formed during incomplete combustion of organic matter, and possess fused aromatic rings (Ravindra *et al.*, 2008). During combustion, small fragments of highly reactive organic molecules fuse into more stable aromatic rings, which can continue to fuse and grow in size (Richter & Howard, 2000). In general smaller particles possess a higher PAH content compared to larger particles, due to a higher surface area with an increased ability to absorb organic content (Sheu *et al.*, 1997). From analysis of PAHs across sites in China, approximately 68.4-84.7% of PAHs were absorbed onto particles below 2μM in aerodynamic diameter (Zhou *et al.*, 2003). A number of factors, such as fuel type, combustion temperature and operating conditions will influence the type of PAHs produced in a vehicle exhaust (Lima *et al.*, 2005). Sixteen 'priority PAHs', as listed in Table 1.1, have been identified by the US Environmental Protection Agency (EPA) due to concern about risks of harm and exposure likelihood (Bojes & Pope, 2007). Efforts have been made to establish various forms of toxic equivalency factors so that the relative risks of each PAH can cross-compared. Although PAH composition may vary in diesel emissions it is possible for the total toxicity equivalent concentration to remain unchanged (Petry *et al.*, 1996; Rojas *et al.*, 2011). The distribution of PAHs on PM of different sizes is dependent on the number of aromatic rings. PAHs possessing two or three rings possessed a bimodal size distribution, with peaks location on PMs <1.1 μm and between 3.3-7.0 μm in size. PAHs possessing more than four aromatic rings had a unimodal size distribution being bound with PM <1.1 μm (Zhou *et al.*, 2003).

As an example, phenanthrene is one of the most abundant PAHs present in the environment (Hwang & Wade, 2008). It has one of the simplest structures with only 3 aromatic rings and also exhibits increased bioavailability due to increased water solubility (Hylland, 2007). Due to its persistence in the environment, studies investigated the effects of phenanthrene in wildlife and found that phenanthrene increases the time for fish embryos to hatch (Horng *et al.*, 2010). Metabolites of phenanthrene measured in urine can be used as a marker exposure to diesel exhaust (Kuusimäki *et al.*, 2004).

Polycyclic Aromatic Hydrocarbon	Number of rings	Polycyclic Aromatic Hydrocarbon	Number of rings
Naphthalene	2	Chrysene	4
Acenaphthene	3	Pyrene	4
Acenaphthylene	3	Benzo(a)pyrene	5
Anthracene	3	Benzo(b)fluoranthene	5
Phenanthrene	3	Benzo(k)fluoranthene	5
Fluorene	3	Dibenz(a,h)anthracene	6
Fluoranthene	4	Benzo(g,h,i)perylene	6
Benzo(a)anthracene	4	Indeno[1,2,3-cd] pyrene	6

Table 1.1: Priority PAHs.

Sixteen PAHs have been selected by the EPA as 'Priority PAHs' to be closely monitored in the environment, due to their potential for biological harm and likelihood for exposure.

1.3.2.3 Metallic components

Although metallic species only make up a small fraction of PM, their presence is still thought to be biologically relevant (Lough *et al.*, 2005). A range of different metallic species have been identified in DEP, including iron, lithium, vanadium and lead (Lim *et al.*, 2009). A study by (Jensen, 2006) also found zinc and lead oxides to be present. Another study demonstrated that titanium and manganese and chromium can also be present (BeruBe *et al.*, 1999). In keeping with other components of DEP, the metal content of DEP has been shown to vary with engine operating conditions (Wang *et al.*, 2003). The sources of metals in DEP can be either lubricant additives or engine wear that occurs during engine operation (Lombaert *et al.*, 2004). When ferrocene is purposely added to diesel fuel, iron rich DEP are formed that increase in size with an increasing concentration of the additive (Lee *et al.*, 2006).

1.3.3 Exposure to DEP

Vehicle emissions represent one of the primary sources of DEP in an urban environment (Fig 1.8). Before outlining the typical levels of DEP that an individual could be exposed to, it is worth noting that in more controlled laboratory settings the particle deposition profile of DEP in the airways of healthy volunteers can vary significantly despite identical doses being inhaled (Rissler *et al.*, 2012). Deposition patterns for COPD patients also differs from healthy individuals (Löndahl *et al.*, 2012). Therefore, it is important to consider that while ambient

concentrations can be quoted for a single sample site, individual exposure doses can be variable.

On the sidewalks of New York City, wearable monitors recorded concentrations of general $PM_{2.5}$ and elemental carbon (a surrogate marker for DEP) across different sample sites. While $PM_{2.5}$ varied only moderately across sample sites, the variation in elemental carbon levels ranged from 1.5μg/m^3 to 6.0μg/m^3 and was associated with heavy vehicle road traffic. (Kinney *et al.*, 2000). Similar studies in London focused on different forms of transport through the city at different times of the year, finding that cyclists were exposed to 11.2μg/m^3 of elemental carbon in the summer but 16.4μg/m^3 in the winter (Adams *et al.*, 2002). This same study also found large differences between heavily trafficked routes compared to quieter routes. When considering distance from a freeway in the City of Los Angeles, the number of ultrafine particulates dropped fairly rapidly and measurements 300m downwind of the roadside were identical to upstream sample locations (Zhu *et al.*, 2002).

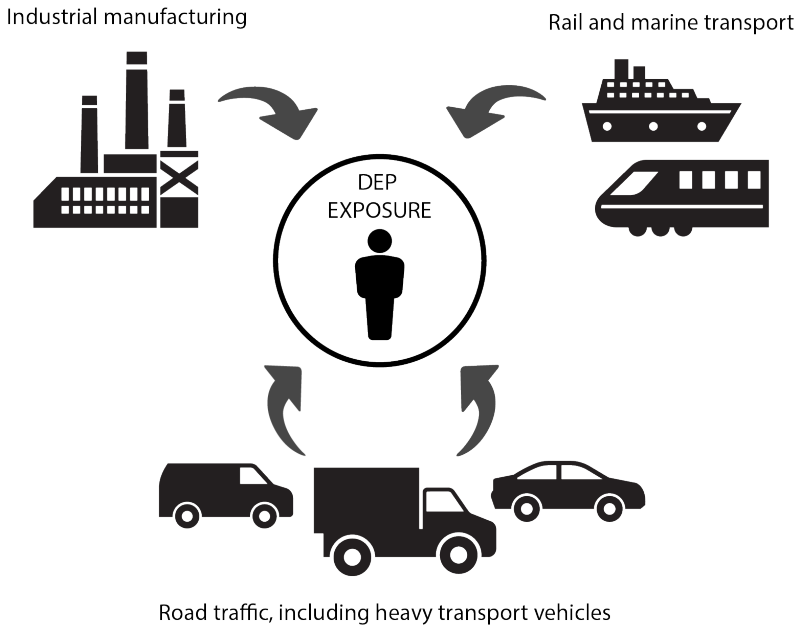


Figure 1.8: Major routes of DEP exposure.

Individuals can be exposed to DEP from a variety of routes. Diesel powered motor vehicles are a significant source of DEP, with higher ambient concentrations found near the roadsides of heavily trafficked roads. Other routes of exposure include DEP produced from industrial manufacturing processes or diesel engines powering train locomotives and marine vehicles.

Exposure to DEP does not just occur when in close proximity to busy roads, but also within motor vehicles themselves. Using in vehicle monitoring systems the highest levels of black

carbon (another marker of DEP) were found within diesel powered vehicles. Averaging out the results, in vehicle black carbon exposure was approximately 6μg/m^3 (Fruin *et al.*, 2004). In the study comparing transportation methods, Adams *et al*., (2002) found that car users were exposed to far higher concentrations of elemental carbon that cyclists or bus passengers. Diesel engines are also frequently found in buses and heavy duty vehicles, and the ambient air found within these vehicles can experience diesel exhaust concentrations that are four times the average concentration of the external environment (Solomon *et al.*, 2001)

As diesel engines are found regularly in an industrial setting, occupational exposure to DEP can be a significant risk for workers who often encounter ambient DEP concentrations far higher than at the roadside (Oberdorster, 2004). Diesel exhaust can be encountered across a wide variety of workplace environments, including railway repair stations, bus garages, warehouses, airport and ambulance depots. (Groves & Cain, 2000). In a review concerning occupational exposure to diesel engine exhaust, those working in enclosed spaces such as miners or other construction workers could encounter concentrations up to 658μg/m^3 (Pronk *et al.*, 2009).

1.3.4 Adverse health effects of DEP

The majority of health effects attributed to PM exposure can also be associated with DEP, strengthening the argument that it is one of the hazardous components of PM (McClellan, 1987; Sydbom *et al.*, 2001; Reed *et al.*, 2004; Ris, 2007; Hesterberg *et al.*, 2010).

As the respiratory system is in intimate contact with the airborne environment, the effects of DEP on the respiratory system will be the focus of this thesis. However, brief mention should be made with regard to the effects of DEP on other physiological systems (Fig 1.9). DEP has been shown to affect the cardiovascular system by increasing the risk of cardiac arrhythmias and myocardial infarction, as well as being associated with hypertension and atherosclerosis (Sydbom *et al.*, 2001). In controlled diesel exhaust exposure studies, diesel exhaust has been reported to induce eye and nose irritation (Rudell *et al.*, 1996) DEP can also affect embryonic development, as *in utero* exposure of DEP in mice resulted in adults with weight gain, altered blood pressure and an increased heart failure susceptibility (Weldy *et al.*, 2014).

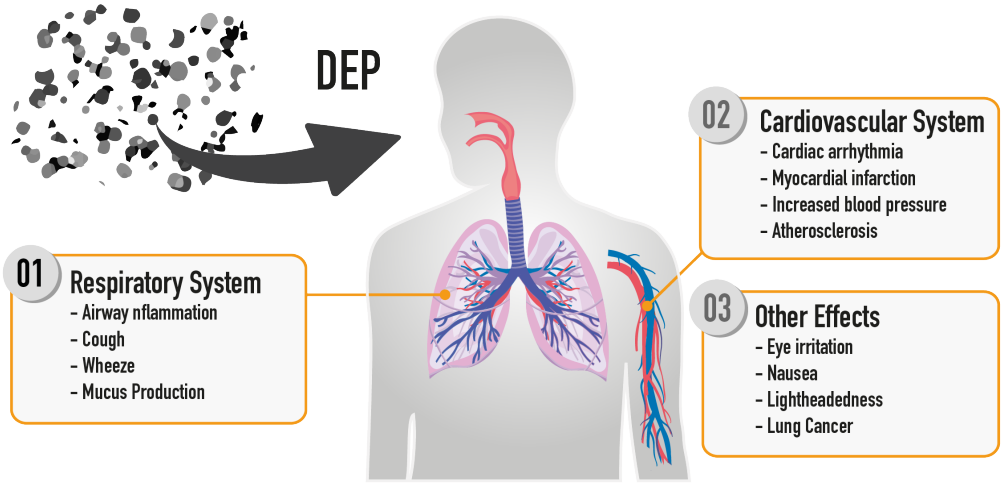


Figure 1.9: Health effects of DEP exposure.

From the results of epidemiological studies, as well as laboratory investigations using human and animal models, exposure to DEP has been shown to have a range of adverse health effects on the cardiovascular system and the respiratory system as well as other effects.

In terms of the respiratory system, long term studies have indicated that exposure to DEP is associated with symptoms such as cough, as well as mucus production and bronchitis (Pronk *et al.*, 2009) In a study of 283 male diesel bus garage workers, approximately 25% of individuals were reported to suffer from chronic cough (Gamble *et al.*, 1987). In children, cough has been associated with various emissions from traffic vehicles (Nicolai *et al.*, 2003). Using questionnaires, respiratory symptoms including cough were reported more frequently in children living in closer proximity to roads with significant truck traffic. In a similar finding, children in closer proximity to heavy road traffic had an increased risk of recurrent dry night cough (Sucharew *et al.*, 2010). However, other studies have reported a negative association between markers of DEP and cough (Patel *et al.*, 2010). In controlled exposures to DEP in adult volunteers, the effects of DEP exposure on lung function is not entirely conclusive. Increases in airway resistance have been reported (Rudell *et al.*, 1996). Several studies show decreases in lung function measurements, such as FVC or FEV_1, however others report negative findings. Variations in exposure profiles and DEP properties may in part explain these discrepancies (Hesterberg *et al.*, 2010).

A prevalence of wheeze in children is associated with DEP exposure (Nicolai *et al.*, 2003), with proximity to traffic compromised of trucks and buses also showing an association of wheeze in toddlers (Bernstein, 2012). Black carbon, a marker of DEP concentration, was also associated with wheeze in adolescences (Patel *et al.*, 2010). Decreases in lung function in

children are associated with exposure to diesel truck emissions, with a lesser association to petrol powered general motor vehicles (Brunekreef *et al.*, 1997). It is concerning that exposure in early life may lead to chronic effects that leave lasting burdens on individuals. However, thankfully policies aiming to reduce pollution levels appear to mitigate developmental impairment, as improvements in air quality result in improvements in lung function development in children (Gauderman *et al.*, 2015).

1.3.5 Effects of DEP exposure in respiratory disease

Several studies have highlighted that individuals with underlying respiratory diseases are more acutely affected by the effects of DEP than healthy individuals.

1.3.5.1 Asthma and allergy

The prevalence of asthma, an inflammatory respiratory condition characterized by the reversible obstruction of airflow, is between 1-18% of the population depending on the country (Bateman *et al.*, 2008). With links between DEP, airway inflammation and bronchoconstriction, it is unsurprising that asthmatic individuals also appear to be particularly sensitive to diesel pollution (Zhang *et al.*, 2009). In a study comparing the effects of walking through an inner city park versus walking by the roadside in close proximity to diesel vehicles, asthmatics remained asymptomatic but showed consistent reductions in FEV_1 and FVC, with greater effects observed in moderate compared to mild asthmatics (McCreanor *et al.*, 2007). Worryingly, exposure to diesel exhaust has been shown to increase airway hyper-responsiveness even in the presence of inhaled corticosteroid therapies (Nordenhäll *et al.*, 2001), indicating that even asthmatics that are managing their disease in the clinically recommended manner may still have an asthma attack triggered by diesel pollution. Children appear to be particularly sensitive to DEP in terms of triggering asthmatic symptoms (Nicolai *et al.*, 2003).

In terms of associations between DEP and allergic responses, DEP exposure has been shown to increase levels of IgE as measured from human test subjects (Diaz-Sanchez *et al.*, 1994; Nel *et al.*, 1998). Controlled exposure of DEP in a laboratory setting also enhanced allergen induced IgE responses in atopic individuals (Carlsten *et al.*, 2016). Dock workers who are consistently exposed to diesel emissions from forklift trucks also have higher IgE serum levels compared to office workers (Mastrangelo *et al.*, 2003). Whether exposure to DEP can increase the likelihood of developing allergic phenotypes remains unclear (Codispoti *et al.*, 2015). 1.0mg/m^3 of DEP enhanced nasal allergic reactions induced by administration of OVA in

guinea pigs (Kobayashi, 2000) Similar results have also have been demonstrated in mice (Takafuji *et al.*, 1987). It is unclear from epidemiological studies whether DEP contributes to the development of allergic diseases, however in the laboratory early life exposure to DEP in mice increased sensitivity to ovalbumin (OVA) (Manners *et al.*, 2013). DEP exposure to pregnant mice also resulted in offspring with reduced IgE production in response to allergen (Corson *et al.*, 2010).

1.3.5.2 Lung cancer

Exposure to diesel exhaust has been shown to increase the risk of lung cancer across several studies, often in occupational settings where individuals can be in extended close proximity to diesel emissions. Exposure to diesel exhaust is also linked to an increased lung cancer risk in U.S. railroad workers (Garshick *et al.*, 2015) and in miners (Attfield *et al.*, 2012). A meta-analysis looking across 23 studies also confirmed a casual association between diesel exhaust exposure and lung cancer risks (Bhatia *et al.*, 1998)

1.3.6 Mechanisms driving adverse effects of DEP

Airway inflammation

Airway inflammation is likely to play a role in the symptoms associated with exposure to DEP. In healthy volunteers, 1 hour of exposure to diesel exhaust during exercise resulted in significant increases in neutrophils, mast cells and other immune cells in the airway lavage fluid as compared to clean air controls (Salvi *et al.*, 1999). These results are in general agreement with others in the literature, highlighting that markers of inflammation or immune system activation in humans remain elevated several hours after DEP exposure (Nightingale *et al.*, 2000; Nordenhäll *et al.*, 2000). It has been noted however from a meta-analysis of available data that very high concentrations of DEP, around 300µg/m^3, are required to induce notable airway inflammation (Ghio *et al.*, 2012b). Increases in neutrophils in bronchial alveolar lavage fluid (BALF) after exposure to DEP in animal models are also reported (Ichinose *et al.*, 1995) It has been shown that around 10% of an inhaled dose of DEP was retained in the rat lung, with the overwhelming majority deposited in the extrapulmonary bronchi (Gerde *et al.*, 2004). Intratracheal installation of DEP that was relatively low in metal content nevertheless increased iron accumulation in the lung, accompanied with increased inflammatory markers in lavage fluid (Ghio *et al.*, 2000).

On a cellular level, DEP releases several key inflammatory cytokines from various cell types found within the lung, including granulocyte macrophage colony-stimulating factor (GM-CSF), interlukin-6 (IL-6) and interlukin-8 (IL-8), in both *in vivo* and *in vitro* models (Steerenberg *et al.*, 1998; Takizawa *et al.*, 1999; Boland *et al.*, 2000; Baulig *et al.*, 2003b; Mazzarella *et al.*, 2007; Holder *et al.*, 2008; Tal *et al.*, 2010). In human bronchial epithelial cells (BEAS-2B) DEP exposure induces cyclooxygenase-2 (COX-2) expression, a gene closely related to inflammatory responses (Cao *et al.*, 2007). Induction of cytokines and other inflammatory mediators has been hypothesised as an underlying mechanism linking DEP to exacerbations in respiratory disease in asthma. Using T-cells obtained from asthmatic patients, when cultured with DEP the proliferation of asthmatic cells occurred at a greater rate compared to controls (Mamessier *et al.*, 2006) and in the same study, DEP exposure increased interferon gamma (IFN-γ) and interlukin-4 (IL-4) production, especially in cells from patients currently experiencing exacerbations. In human bronchial epithelial cells, high concentrations of DEP (50-100 µg/ml) were required to release inflammatory cytokines in healthy patients. However, lower concentrations could elicit responses in cells from asthmatic patients (Devalia *et al.*, 1999). In mice, the ability of DEP to be phagocytosed appears to influence whether a Th1-type or Th2-type inflammatory response is initiated (Park *et al.*, 2011) Inhalation of DEP in asthmatic patients increased inflammatory neuropeptide release (Sava *et al.*, 2013). *In vitro* studies have also investigated the ability for DEP to induce cell death, which would also contribute to overall airway inflammation. Although certain studies have highlighted cytotoxicity in cultured cells (Matsuo *et al.*, 2003a; Danielsen *et al.*, 2008), others report that the addition of DEP to not cause cell death (Don Porto Carero *et al.*, 2001). Variations in experimental protocol may explain these discrepancies, as engine operating conditions have been shown to alter cytotoxicity (Knebel *et al.*, 2002).

Immune System

DEP have also been show to interfere with the function of the immune system. In T-lymphocytes, DEP exposure has been shown to impair autophagic-lysosomal mechanisms as well as altering cytokine expression profiles. (Pierdominici *et al.*, 2014). Pulmonary dendritic cell recruitment and maturation within the lung are also modulated by DEP exposure (Provoost *et al.*, 2010). Macrophages cultured in the presence of DEP exhibited mitochondrial and lysosomal dysfunction, a gradual decrease in cell numbers over time and impaired cytokine response to lipopolysaccharides (LPS). (Chaudhuri *et al.*, 2012) (Verheyen *et al.*, 2004) reported dysregulation of a wide range of genes after exposure to DEP in macrophages derived from the human monocytic THP-1 cell line. Bacterial clearance by macrophages is also slowed by DEP exposure (Yin *et al.*, 2002). While DEP may increase cytokine production

in certain instances, in alveolar macrophages it has also been shown to impair IL-8, tumour necrosis factor alpha (TNF-α) and prostaglandin E_2 (PGE_2) in response to LPS stimulation (Mundandhara *et al.*, 2006).

Oxidative Stress

The ability to generate oxidative stress has been proposed as one of the major mechanisms by which DEP can induce adverse health effects (Hirano *et al.*, 2003; Pourazar *et al.*, 2005). Oxidative stress describes an abnormal redox state in a biological system whereby the presence of excessive oxidants overwhelm antioxidant defences (Sies, 1997; Kohen & Nyska, 2002). Compounds that can accept electrons are known as oxidizing agents while those that can donate electrons are known as reducing agents. Reduction and oxidation always occur simultaneously given that agents are required to react with each other. In biology, so called redox (reduction-oxidation) reactions influence a range of cellular pathways and an ongoing balance of oxidants and anti-oxidants must be maintained. Any changes toward pro-oxidant or pro-antioxidant states can have negative effects on cellular functions, therefore there is tight regulation of the redox state of the cell. Oxidant stress leads to the release of inflammatory mediators and cytokines, causing widespread cell and tissue damage.

While oxygen is an essential molecule for sustaining life, it can also be highly toxic due to its ability to act as an oxidising agent. Reactive oxygen species (ROS) are some of the main oxidising agents involved in this process, with excessive ROS capable of indiscriminately reacting with phospholipids, proteins and nucleic acids to cause harm (Yu, 1994). ROS can be classified into two groups, free radicals and non-radical oxidisers. Free radicals possess one or more unpaired electrons and are capable of an independent, if short-lived, existence. The presence of an unpaired electron makes them highly reactive, readily accepting or donating an electron in an attempt to become a more stable molecule (Kohen & Nyska, 2002). Examples of free radicals include superoxide ($O_2^{\cdot -}$) or hydroxyl ($OH^{\cdot}$). Superoxide has a relatively long half-life that allows it to diffuse across cellular membranes. In contrast to superoxides, hydroxyl radicals have extremely short half-lives and generally reduce substances in the immediate vicinity of origin. Non-radical oxidisers include hydrogen peroxide (H_2O_2) and ozone (O_3) that can be easily converted into free radicals (Bayr, 2005). To protect against the harmful effects of ROS, cells possess antioxidant defences. Detoxification can occur either by low molecular weight antioxidants (LMWA) or antioxidant enzymes. Enzymes, such as superoxide dismutase (SOD), catalase and peroxidase, enable the conversion of free radicals to H_2O. (Andreyev *et al.*, 2005). Scavenging LMWA donate electrons and prevent oxidants from reacting with biological targets. Reduced glutathione (GSH) is one of the key

LMWA present in a cell and its antioxidant properties can protect the lungs from inflammatory disease states (Rahman & MacNee, 2000)

There are several potential sources of both endogenous and exogenous ROS in a cell. One major source of endogenous ROS are the mitochondria, where superoxides are constantly formed as part of the electron transport chain process when oxygen is reduced to water to produce adenosine triphosphate (ATP) (Murphy, 2009; Sena & Chandel, 2012). Immune cells, such as neutrophils and eosinophils, also produce H_2O_2 and $OH^{\cdot}$ as a means to destroy bacteria (Weiss *et al.*, 1981). Exogenous ROS can be produced from ionising radiation, drugs and air pollutants, including cigarette smoke and vehicle exhaust. Several characteristics of DEP make it capable of generating oxidative stress. As a nanoparticle, the physical size of DEP enable them to enter into cells and even penetrate mitochondria (Li *et al.*, 2003). Mitochondrial damage results in disruption of the electron transport chain and the production of mitochondrial ROS (Sena & Chandel, 2012). Nanometre sized DEP also have an extremely high surface area to mass ratio. A large surface area allows numerous contaminants to be embedded onto its surface. Transition metals are present within DEP, including Fe, Ni, Cu, Co and Cr, and are capable of catalysing reactions to generate ROS (Ball *et al.*, 2000).

Experimentally, it has been confirmed that DEP can generate oxidative stress in both *in vitro* and *in vivo* (Ghio *et al.*, 2012a). In human bronchial epithelial cells, DEP exposure increased intracellular peroxide production resulting in a pro-oxidant state (Baulig *et al.*, 2003b). Generation of ROS and depletion of GSH has been hypothesised to be an underlying mechanism for the sensitising effect of DEP for allergic responses (Casillas *et al.*, 1999; Dong, 2005) Induction of oxidative stress also activates a range of redox sensitive transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) (Pourazar *et al.*, 2005). Matrix metalloproteinase-1 (MMP-1) is also thought to be upregulated in lung epithelial cells via a redox sensitive mechanism (Amara *et al.*, 2007) The phagocytosis of DEP by macrophages results in the generation of reactive oxygen radicals, initiating cell signalling events, deoxyribonucleic acid (DNA) damage and apoptosis (Jantzen *et al.*, 2012). Oxidant stress has also been confirmed through the use of antioxidants to abolish biological responses to DEP. The co-application of the antioxidant N-acetylcysteine (NAC) can prevent inflammatory responses and airway hyper responsiveness to diesel exhaust in both mice and humans (Banerjee *et al.*, 2009; Carlsten *et al.*, 2014). In human bronchial epithelial cells, DEP induced cell death could be prevented with ethyl reduced glutathionate, another antioxidant (Matsuo *et al.*, 2003b).

Interestingly, the organic components have been heavily implicated as the critical DEP components of this oxidative stress response (Hiura *et al.*, 1999; Li *et al.*, 2002). PAHs constitute the majority of the organic components in DEP, and are classical ligands of the aryl hydrocarbon receptor (AhR). Activation of AhR can induce transcription of the enzyme cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1), which ultimately metabolises PAHs to detoxify them. However, during this process intermediary steps involve the formation of reactive quinones that can form ROS. The downstream effects of AhR are not just relegated to the production of ROS, as a wide variety of target genes are capable of being upregulated following the binding of ligands to AhR and these may also be responsible for some of the adverse effects of DEP exposure.

Activation of the Aryl Hydrocarbon Receptor.

As environmental toxins, PAHs are thought to mediate their harmful effects through the activation of the AhR, an intracellular ligand-activated transcription factor (Hahn, 2002). AhR is highly conserved across species, and at basal state is bound to molecular chaperone complexes (Rowlands & Gustafsson, 1997). However, upon ligand binding, AhR translocates to the nucleus and heterodimerises with aryl hydrocarbon nuclear translocator (ARNT), which then initiates the expression of a wide range of genes that are linked with detoxification, including CYP1A1, CYP1A2, CYP1B1 (Lin *et al.*, 2003). Ultimately activation of this pathway is thought to be responsible for the harmful effects of PAHs present in environmental pollutants (Tuyen *et al.*, 2014) Activation of AhR is thought to play a role in asthma and COPD, through increasing mucin production and upregulation of various inflammatory pathways (Chiba *et al.*, 2012).

While some studies have also found that the organic components of DEP are critical for cellular responses (Siegel *et al.*, 2004), in a study by (Pan *et al.*, 2004)) it was noted that cleaned DEP with the organic components removed could still generate ROS. DEP demonstrate AhR ligand activity in mouse hepatoma cell lines (Misaki *et al.*, 2008). DEP that contain PAHs have been shown to compete with 2,3,7,8-tetrachloro[1,6-[3H]]dibenzo-p-dioxin (TCDD – the canonical AhR ligand) in AhR binding in the cytosol of rat liver cells (Soontjens *et al.*, 1997) and induce expression of CYP1A1 and other downstream targets, including inflammatory cytokines (Totlandsdal *et al.*, 2015). The organic compounds of DEP, not the residual carbon core of DEP, are the critical component for induction of CYP1A1 (Totlandsdal *et al.*, 2012). DEP are therefore thought to be able to influence the transcription and translation of a range of environmental toxin response genes.

1.4 Airway sensory nerves

With a current focus on inflammation and immune system driven responses, there is another important aspect of lung physiology that may be influenced by DEP. As previously mentioned, the lungs and airways are in intimate contact with the ambient air, and therefore the body requires the ability to detect and respond to a noxious external environment if the airways may be potentially at risk. This ability is enabled through the actions of airway sensory nerves that innervate the lungs, and it is now clear that they play a role in both health and disease (Belvisi, 2003a; Canning, 2011; Raemdonck *et al.*, 2012; O'Neill *et al.*, 2013; Bonvini *et al.*, 2015).

1.4.1 Anatomy and classification

The majority of airway sensory nerves are housed within the vagus nerve (Cranial Nerve X), with the neuronal cell bodies housed within either the jugular or nodose ganglion that are located just below the ear bone at the base of the skull (Springall *et al.*, 1987). In terms of embryonic origin, the jugular ganglion are derived from the neural crest while the nodose ganglia are derived from the epibrancial placode (Nasra & Belvisi, 2009). Although airway sensory nerves project from two separate origins, neuronal projections from both ganglia extend peripherally to innervate the airways, forming dense plexus of nerve fibres just below the epithelial layer (Kummer *et al.*, 1992). Centrally, the projections extend to the Nucleus Tractus Solitarious (NTS) within the brain and synapse on 2nd order interneurons in the dorsomedial region of the medulla (Mazzone, 2002; Belvisi, 2003b; Kubin *et al.*, 2006)

Airway sensory nerve classification has become increasingly complex (Mazzone, 2005). Using a range of characteristics including anatomical origin/termination, myelination, physicochemical sensitivity, adaptation indices and conduction velocities several subtypes of airway sensory nerves have been identified (Adcock *et al.*, 2014). In a general sense, airway sensory nerves can be categorised as either primarily mechanically sensitive A-δ fibres afferents that respond to the movement of the lung during inflation and deflation or C-fibre afferents that are more responsive to chemical stimuli (Coleridge & Coleridge, 1984b; Belvisi, 2003a; Canning, 2011; Birrell *et al.*, 2014).

A-δ fibres are generally myelinated, possess faster conduction velocities and characteristically respond to mechanical stimuli. Several types of A-δ fibres exist. Rapidly adapting receptors (RARs), as can be inferred from their name, will quickly cease firing over the course of a sustained mechanical stimulus (Sant'Ambrogio & Widdicombe, 2001). Anatomically, the cell

bodies of RARs are thought to be primarily housed in the nodose ganglion (Ricco *et al.*, 1996). With conduction velocities of over 13m/s, RARs are though to play a defensive role by responding to lung hyperinflation or deflation (Canning *et al.*, 2006). A subset of RARs have been identified that also respond to chemical stimuli. (Sant'Ambrogio & Widdicombe, 2001; Adcock *et al.*, 2014) Activation of RARs can cause a cough reflex response even in anesthetised animal, providing a basic protective reflex to clear the lungs (Nasra & Belvisi, 2009; Dicpinigaitis *et al.*, 2014) Slowly adapting receptors (SARs) are similar to RARs but conduct action potentials more quickly (18m/s) and are slow to cease firing in response to a sustained mechanical stimuli. It is thought that these receptors are involved with sensing of tidal breathing pattern, particularly with the stretch reflex that prevents hyperinflation of the lung (Schelegle, 2003). Another fibre subtype that shows some similar characteristics to RARs are A-δ nociceptors. Possessing similar action potential conduction speeds of around 4-6m/s, they are less responsive to mechanical stimuli and have increased chemical stimuli. (Ricco *et al.*, 1996; Yu *et al.*, 2005). Cough receptors are another subtype of A-δ fibres, classified by sensitivity to acid/pH but unresponsive to mechanical stimulation other than light touch and chemical stimuli such as capsaicin (Canning *et al.*, 2004)

In contrast, C-fibres are unmyelinated, with slower conduction velocities of less than 1m/s. While it is possible for C-fibres to be activated by extreme mechanical stimuli, they generally demonstrate acute sensitivity to chemical stimuli. Irritants such as capsaicin, the potent ingredient in chilli peppers, or acrolein, a noxious chemical found in cigarette smoke, are classical activators of C-fibres (Canning *et al.*, 2006; Birrell *et al.*, 2009; Bonvini *et al.*, 2015). Endogenous substances such as bradykinin and PGE_2 are also capable of activating the vagus nerve (Grace *et al.*, 2012). Two populations of C-fibres can be classified as either ‘bronchial’ or ‘pulmonary’ depending on their termination site (Coleridge & Coleridge, 1984a). Another characteristic of C-fibres is their ability to synthesis neuropeptides when activated (Lundberg *et al.*, 1983; Chuaychoo *et al.*, 2005). Such neuropeptides include substance P which can induce bronchoconstriction in the airways (Nasra & Belvisi, 2009).

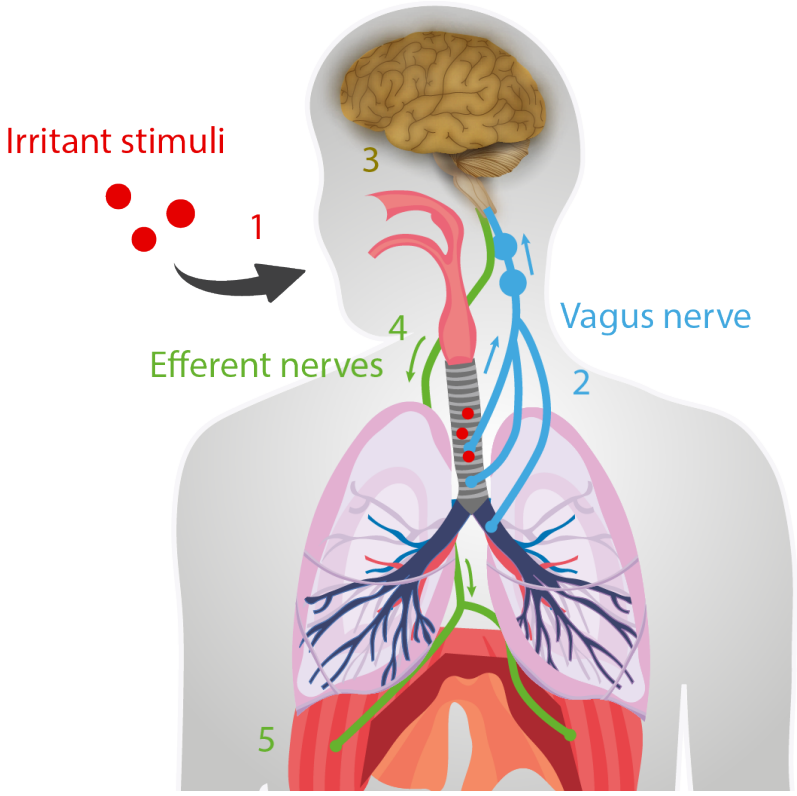


Figure 1.10: Airway sensory nerve reflex pathway.

1. An irritant stimulus is inhaled into the airways. 2. Airway sensory nerve terminals detect the presence of the stimuli and activate the nerve, sending an action potential signal up the vagus nerve and past the nodose and jugular ganglia that house the neuronal cell bodies. 3. The afferent signal is processed in the NTS region of the brain 4. The efferent arm of the signal travels back down to the periphery, either by motor neurons to evoke cough or the parasympathetic system to cause effects such as bronchospasm and mucus secretion 5. Respiratory reflex responses occur, such as cough through the recruitment and activation of respiratory muscles; or bronchoconstriction through the contraction of airway smooth muscle.

1.4.2 Airway sensory reflexes

Activation of airway sensory nerves can mediate several different reflexes that are intended to protect the lungs and airways from harm (Brouns *et al.*, 2012) (Fig 1.10). For healthy individuals, these defensive reflexes are normally beneficial and well tolerated. However, for those with underlying respiratory diseases these reflexes may be triggered inappropriately and be extremely troublesome (McGarvey *et al.*, 2009; Nasra & Belvisi, 2009).

1.4.2.1 Cough

One of the most well studied respiratory reflexes triggered by airway sensory nerve activation is the cough reflex. This is due not only to the robustness of the response that facilitates experimental observation, but also the fact that cough itself is the most common cause for patients to visit a doctor (Schappert & Rechtsteiner, 2011) (Smith *et al.*, 2008). In respiratory disease the cough reflex can be triggered inappropriately and lead to individuals suffering from excessive coughing that can impact their quality of life. Despite this prevalence, current cough treatment medication is unsatisfactory in its efficacy.

Coughing is normally a defensive forced expiratory manoeuvre designed to clear the larynx, trachea and large bronchi of inhaled physical or chemical irritants (Widdicombe, 1995; Fontana *et al.*, 1999). Cough stimuli can be endogenous or exogenous in origin, such as inflammatory mediators like PGE_2 and bradykinin or cigarette smoke and cold air. (Tiittanen *et al.*, 1999; Canning, 2008; Grace & Belvisi, 2011; Grace *et al.*, 2012; Smit *et al.*, 2012). Mechanical stimulation is also capable of triggering cough (Canning *et al.*, 2006).

The cough reflex differs from other airway reflexes as it occurs in a threshold dependent manner (Canning & Mori, 2011)}. Tussive stimuli must activate a sufficient number of receptors on sensory nerve endings to achieve the required level of neuronal depolarisation to generate an action potential that will propagate up the vagus nerve. After being processed centrally, the signal is passed down efferent motor neurons to stimulate contractions in respiratory muscles such as the diaphragm to lead to the forced respiratory event that is cough. Cough occurs in three distinct phases, firstly rapid inhalation, then closing of the glottis to increase intrathoracic pressure and then finally the expiratory phase whereby air is rapidly expelled and the characteristic cough sound is generated (Bianco *et al.*, 1988; Smith, 2014)

Cough is a significant burden for patients in society.(Schappert & Rechtsteiner, 2011). Cough can either be defined as acute (< 3 weeks in duration) or chronic (> 8weeks in duration) (Irwin *et al.*, 1998). When just considering clinical causes, acute cough is normally attributed to specific bacterial or viral infections (Curley *et al.*, 1988; Irwin *et al.*, 1998). Chronic cough usually manifests in long standing respiratory diseases. In asthma, a condition defined as a chronic airway inflammatory disease with reversible bronchoconstriction, the majority of patients suffer from cough as a symptom (Pavord, 2004; Rodrigo *et al.*, 2004; Bateman *et al.*, 2008; Desai & Brightling, 2010). Another respiratory disease that is closely linked with cough is COPD. COPD is also characterized with chronic airway inflammation driven by chronic bronchitis, bronchiolitis and emphysema (Barnes, 2000; Mannino & Buist, 2007). There is a

close relationship between aging and COPD, as well as a history of smoking (Sethi & Rochester, 2000; Ito *et al.*, 2004). Other diseases associated with cough include pulmonary fibrosis (Hope-Gill *et al.*, 2003; Key *et al.*, 2010), post-nasal drip (Irwin *et al.*, 1984), gastroesophageal reflux disease (Novitsky *et al.*, 2002; Poe & Kallay, 2003) and cough can also be a side effect of angiotensin-converting-enzyme (ACE) inhibitor medication (Wood, 1995). Additionally, a certain percentage of the percentage of the population also suffers from idiopathic cough, defined as cough that has no known set origin and yet patients present with hypersensitivity to cough stimuli (McGarvey & Ing, 2004; Morice, 2010). Whatever the cause, chronic cough can have significant negative impacts on the quality of life of the sufferer through sleep disturbance, nausea and urinary incontinence (French *et al.*, 1998; 2002; Lee & Birring, 2010). Additionally, social relationships are also affected and patients can also suffer psychological distress (Dicpinigaitis *et al.*, 2006; Brignall *et al.*, 2008).

1.4.2.2 Bronchoconstriction and mucus secretion

Activation of airway sensory nerves has been shown to induce other reflexes such as bronchoconstriction (Forsberg *et al.*, 1988) and mucus secretion (Kuo *et al.*, 1990). These neuronal mechanisms have been demonstrated to be mediated by alternative efferent pathways compared to cough. The cholinergic parasympathetic nervous system is involved and controls airway smooth muscle tone. Acetylcholine binding to nicotinic acetylcholine receptors mediates the synaptic transmission of signals received centrally to the necessary parasympathetic ganglia within the airway smooth muscle (Myers, 2001). Activation of muscarinic receptors leads to bronchoconstriction, mucus secretion and bronchial vasodilation(Barnes, 1995).

Activation of C-fibre afferents releases also neuropeptides such as calcitonin gene-related substance P and neurokinin A (Karlsson, 1993) as a local reflex. Such neuropeptides are potent regulators of airway smooth muscle contraction and can induce vasodilation. Additionally, neurokinins can also induce bronchial oedema and mucus secretion. These effects are mediated by neurokinin receptor 1 and neurokinin receptor 2 that are expressed on a multitude of cell types within the lung. Application of neurokinin receptor antagonists have been shown to inhibit bronchoconstriction and mucus secretion evoked by electrical and chemical stimuli (Maggi *et al.*, 1991; Lei *et al.*, 1992) . It must be noted that these neurokinin mediated pathways appear to be more robust in animal models such as the guinea pig, and their relevance in man is debated. Previous experiments using neurokinin receptor

antagonists in humans were unable to prevent neurokinin A induced bronchoconstriction in asthmatics (Joos *et al.*, 1987), but more recent tools may provide new insights.

1.4.3 Airway sensory nerve activation

To initiate airway reflexes, airway sensory nerves must first receive some appropriate stimuli, whether mechanical or chemical in nature. Ion channels on the surface of the nerves then allow an influx of ions into the cell and lead to the initial depolarisation of the neuronal membrane, also known as the generator potential (Taylor Clark & Undem, 2006). If the generator potential reaches a sufficient magnitude, it triggers classical action potential firing. At the start of an action potential, membrane depolarisation triggers the 'all or nothing' opening of voltage-gated sodium channels (VGSCs) (Catterall, 2000). Following an influx of Na^+, the neuronal membrane shifts from a resting potential of -70mV to a less negative value and thus further depolarizes. A wave of depolarisation then propagates along the vagus nerve as adjacent VGSCs open in response to activation of their neighbour, leading the generation of the classical action potential signal. After opening, VGSC quickly inactivate after a few milliseconds until the membrane repolarises (Catterall, 2000) , which occurs through the opening of voltage gated potassium channels (VGKCs). After a brief period of overshoot, the VGKCs close and a resting membrane potential of -70mV is restored. The nerve is then ready to generate another action potential. These signals travel up the vagus nerve, where they are processed in the relevant respiratory control centres of the brain before reflex signals are sent back to the periphery, either driving parasympathetic reflexes or motor neuron driven reflexes(Kubin *et al.*, 2006; Nasra & Belvisi, 2009). In the case of cough, multiple high frequency action potential signals are required before the cough reflex is initiated and are responsible for the sensations of an urge to cough that proceeds the full cough reflex event (Canning & Mori, 2011).

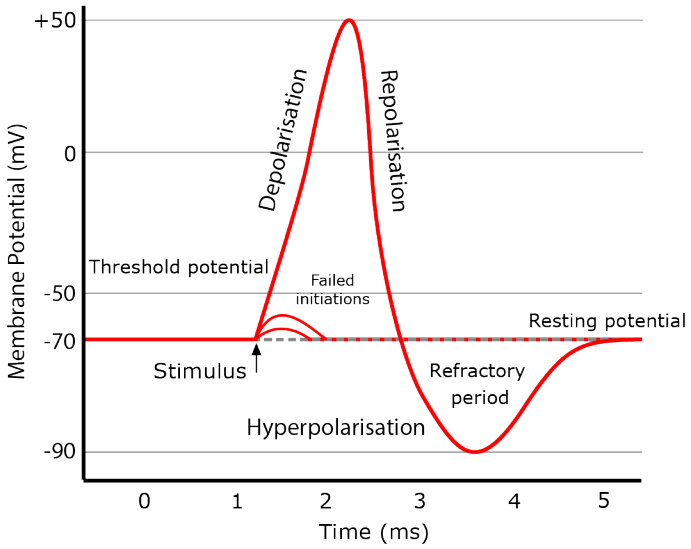


Figure 1.11: The neuronal action potential.

The resting membrane potential of a neurons is around -70mV. If sufficient depolarisation to a threshold potential (-50mV) occurs the 'all or nothing' action potential signal is initiated. VGSCs open and allow Na^+ into the neuron, depolarising the cell to around +50mV. At this point, VGSCs close and VGKCs open and the resulting K^+ flux repolarises the cell. During an overshoot period, the cell hyperpolarises and remains in a refractory period until the resting potential is reached again. At this point the neuron has recovered and is ready to fire another action potential. Note that if the threshold potential is not reached, then the neuron quickly returns to the resting membrane potential.

A number of ion channels are capable of generating the depolarisation required to initiate an action potential in airway sensory nerves. Purinergic P2X channels can be activated by the global signalling molecule ATP, through the binding of ATP to the extracellular sections of the channel (Khakh & North, 2006). During inflammation and fibrosis, ATP is widely released from multiple cell types in the lung and is thought to act as a 'danger signal' (Bodin & Burnstock, 1998; Idzko *et al.*, 2007; Riteau *et al.*, 2012). ATP can activate airway sensory nerve fibres from the nodose ganglia and this activation can be blocked by a selective P2X3 antagonist A-317492 (Kwong *et al.*, 2008). Activation of airway sensory nerves by ATP has also been shown to cause cough in guinea-pigs and cough and dyspnoea in asthmatics and COPD patients (Basoglu *et al.*, 2005; 2015). Through this ATP-P2X3 pathway, airway sensory nerves are capable of detecting and responding to an endogenous 'danger signal'. In addition to the purinergic ion channels, transient receptor potential ion channels (TRPs) are another important superfamily of receptors expressed on airway sensory nerves that respond to a far greater variety of stimuli than just ATP.

1.4.4 Transient Receptor Potential (TRP) Ion Channels

The superfamily of TRPs play important roles as environmental sensors on airway sensory nerves and the generation of sensory reflexes (Clapham, 2003). TRP channels were first identified in the Dropsophilia fly, and named after a transiently observed response to bright light (Montell & Rubin, 1989) Structurally, TRP channels are six membrane spanning cation channels that possess an intracellular N and C terminus and a variable number of ankyrin repeats. A cation permeable pore exists between transmembrane domains 5 and 6, which allows an influx of cations into the cell when the channel is activated (Pedersen *et al.*, 2005).

TRP channels are activated by a wide variety of stimuli, including chemicals, temperature, osmolality or mechanical stress (Bonvini *et al.*, 2015). The majority of TRP channels are calcium permeable, although some of them are permeable to other specific ionic species such as Mg^{2+} (Pedersen *et al.*, 2005). In neurons, activation of certain calcium permeable TRPs leads to an influx of Ca^{2+} into the cell and the initial depolarisation that leads to action potential generation (Fig 1.12). There are 7 subfamilies of TRPs: TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, and TRPV (Pedersen *et al.*, 2005). Of these TRPs, several have been shown to be expressed on airway sensory nerves and can induce respiratory reflexes upon their activation.

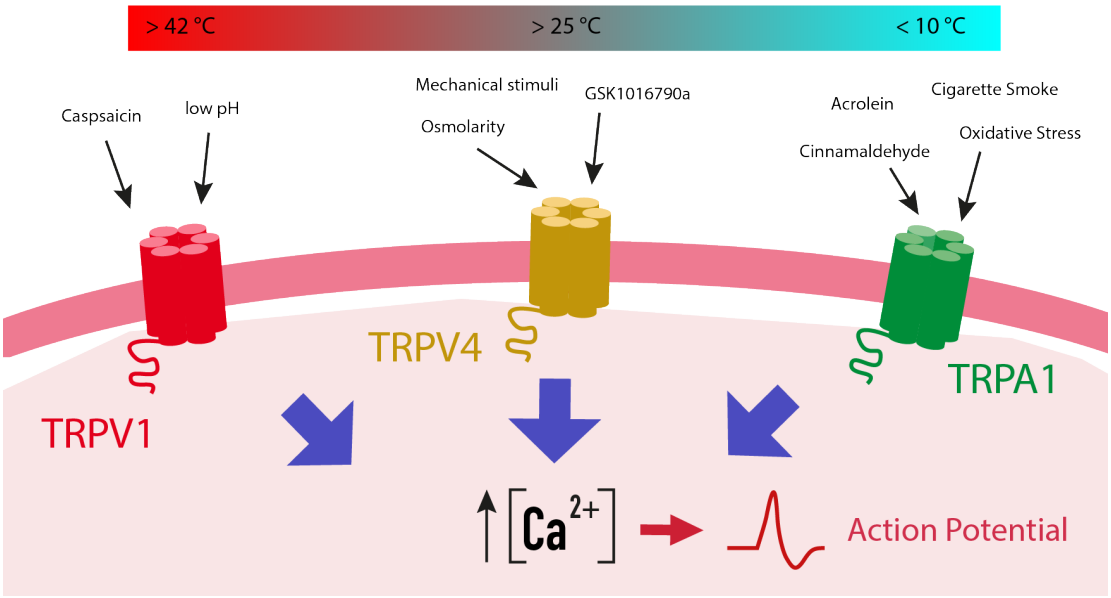


Figure 1.12: TRP channels expressed on airway sensory nerves.

Several TRPs expressed on airway sensory nerves are capable of initiating respiratory reflexes. TRPV1 can be activated by high temperatures (>42˚C), as well as capsaicin and low pH. TRPV4 is a

mechanosensor and osmolarity sensor, that can also be activated by moderate physiological temperature and synthetic small molecules. TRPA1 can be activated by a wide range of irritant stimuli, including both endogenous inflammatory mediators and environmental pollutants, as well as cold temperatures (<10˚C). Activation of these TRP channels has been shown to increase intracellular calcium and generate action potentials within the vagus nerve that initiate respiratory reflex events such as cough.

Transient Receptor potential Vanilloid 1 (TRPV1)

TRPV1 was the first TRP channel to be cloned and characterized by patch clamp electrophysiology (Caterina *et al.*, 1997). It is commonly known as a nociceptor responsible for detecting noxious heat and is classically activated by capsaicin, the active component compound found within hot chilli peppers. TRPV1 has attracted significant attention in the pain research field (Immke & Gavva, 2006), as it highly expressed on peripheral and central sensory nerves. However, it was also the first TRP channel to be identified to play a key role in respiratory reflexes (Lalloo *et al.*, 1995).

Structurally, TRPV1 contains a vanilloid binding pocket between transmembrane regions 3 and 4. In this region several residues have been identified that regulate the channel's activation and are also sites where many TRPV1 antagonists bind (Immke & Gavva, 2006). TRPV1 can be activated by a diverse range of stimuli including endogenous and exogenous mediators (Macpherson *et al.*, 2005; McNamara *et al.*, 2005; Adcock, 2009; Raisinghani *et al.*, 2011). The classic agonist of TRPV1 is capsaicin. Other agonists include resinoferotoxin (RTX) and retinoid compounds that are structurally related to vitamin A (Yin *et al.*, 2013). Indirect activators including high temperatures (>42˚C) and low pH, are thought to sensitise the channel and increase the probably of channel opening (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). The pore opening of TRPV1 can also be regulated by the activation of linked G-coupled protein receptors (GPCRs) by endogenous mediators such as bradykinin and PGE_2 (Grace *et al.*, 2012). Phosphorylation of protein kinase A and protein kinase C is also capable of modulating TRPV1 activity (Bhave *et al.*, 2002; Numazaki *et al.*, 2002).

With respect to the lungs, TRPV1 is expressed in a number of cell types including smooth muscle cells, bronchial and tracheal epithelial cells (Zhao *et al.*, 2013; McGarvey *et al.*, 2014). TRPV1 is also widely expressed in neuronal cells including vagal ganglia (Caterina *et al.*, 1997; Grace *et al.*, 2012; Lieu *et al.*, 2012). Capsaicin and other TRPV1 ligands have been shown to activate the airway sensory nerves both *in vivo* and *in vitro* (Canning *et al.*, 2006;

Bessac & Jordt, 2008; Adcock, 2009; Grace *et al.*, 2012). Inhalation of TRPV1 ligands can trigger the cough reflex both in animals and in man. The response to capsaicin is robust and repeatable, and it is used clinically as a safe challenge for patients to assess sensitivity of the cough reflex (Dicpinigaitis, 2003; Smith, 2014). In addition to coughing, activation of TRPV1 can lead to sneezing, mucus secretion and pain (Geppetti *et al.*, 1988; Tominaga *et al.*, 1998). The release of neuropeptides such as substance P from C-fibre afferents in response to TRPV1 activation activates neurokinin receptors found on airway smooth muscle leading to bronchoconstriction (Hunter & Undem, 1999; Cho, 2003; De Swert & Joos, 2006). TRPV1 expression and activation also appear to be altered in respiratory diseases, highlighting its important role in the lungs and airways. An increased sensitivity to capsaicin is found in diseases such as asthma and COPD, indicating that the prevalence of cough in these respiratory diseases has important neuronal mechanisms (Choudry & Fuller, 1992; Belvisi *et al.*, 2016; Marsden *et al.*, 2016; Satia *et al.*, 2016).

Transient Receptor potential Vanilloid 4 - TRPV4

TRPV4 is a polymodal receptor that was first identified as being sensitive to osmotic stress (Liedtke *et al.*, 2000). TRPV4 contains a proline rich region with 6 ankyrin repeats within its N-terminus (Everaerts *et al.*, 2010) TRPV4 is widely expressed throughout the lung, including epithelial cells, smooth muscle and macrophages (Lorenzo *et al.*, 2008; Hamanaka *et al.*, 2010). TRPV4 ligands have been only shown to stimulate nodose ganglia neurons (and not jugular) and activate A- δ and not C-fibres (Bonvini *et al.*, 2016).

TRPV4 can be activated by both changes in mechanical stretch and osmolality, as well as relatively moderate temperatures of around 24˚C (Strotmann *et al.*, 2000; Gao *et al.*, 2003; Suzuki *et al.*, 2003), although the mechanisms of activation are thought to be different for each (Vriens *et al.*, 2004). In terms of ligands, synthetics small molecules, such as GSK1016790a (GSK101), and phorbol esters, such as 4α-phorbol 12,13-didecanoate (4αPDD), have also been shown to activate TRPV4 (Watanabe *et al.*, 2002; Bonvini *et al.*, 2013).

The functional role of TRPV4 activation has not been as thoroughly investigated as TRPV1 or TRPA1 activation. However, GSK101 induces cough within the conscious guinea pig which can be blocked by the selective TRPV4 antagonists (Bonvini *et al.*, 2013; 2016). Agonists also have been shown to cause bronchoconstriction through the release of cysteinyl leukotrienes (Bonvini *et al.*, 2016). Proteases such as neutrophil elastase have been proposed as endogenous ligands for TRPV4 (Zhao *et al.*, 2015). Hypotonic solutions have been reported

to cause cough in asthmatic patients (Fuller & Collier, 1984). Several SNPs of TRPV4 have also been found to be associated with COPD (Zhu *et al.*, 2009).

Transient Receptor potential Ankyrin 1 (TRPA1)

TRPA1 is Ca^{2+} permeable ion channel that can be activated by a range of natural substances and chemical irritants (Bandell *et al.*, 2004; Bautista *et al.*, 2005; Trevisani *et al.*, 2007; Taylor-Clark & Undem, 2010). It was first identified by its ability to detect noxious cold temperatures and is the only known mammalian member of the ankyrin TRP family (Clapham, 2003; Story *et al.*, 2003).

TRPA1 is widely expressed in neuronal tissue. Within the vagal ganglia cells, the reported fraction of neurons that express TRPA1 within the jugular and nodose ganglia are roughly around 30%(Story *et al.*, 2003). Several studies also have found that TRPA1 is co-expressed with TRPV1 (Anand *et al.*, 2008; Salas *et al.*, 2009) although TRPA1 ligands only activate C-fibre afferents, not A-δ afferents using *in vivo* guinea pig airway single fibre firing(Adcock *et al.*, 2014). When considering non-neuronal cells, TRPA1 has been found to be expressed within lung fibroblasts, alveolar epithelial cells, bronchial epithelial cells and smooth muscle cells(Fernandes *et al.*, 2012).

TRPA1 contains 14 ankyrin repeats in its N terminus, which is unique among the other TRP channels (Cordero-Morales *et al.*, 2011). Due to its molecular structure, TRPA1 is sensitive to activation by electrophiles that can covalently modify exposed cysteine residues within the N-terminus region (Macpherson *et al.*, 2007). This method of activation is the major mechanism by which ligands of TRPA1 can activate the channel, although like other TRP channels, TRPA1 can be modulated by GPCRs such as the bradykinin receptor and protease activated receptor 2 (PAR_2), as well as calcium levels (Bandell *et al.*, 2004; Dai *et al.*, 2007; Zurborg *et al.*, 2007). In addition to ligand activation, TRPA1 can also be activated by noxious cold below 10˚C (Karashima *et al.*, 2009; del Camino *et al.*, 2010).

When considering direct activators, a large number of TRPA1 ligands are structurally distinct but share highly reactive electrophilic carbon moiety as a common characteristic (Macpherson *et al.*, 2007; Bang & Hwang, 2009). These ligands are capable of covalent binding to lysine and cysteine residues on TRPA1 to activate the channel. Well known examples of TRPA1 ligands include allyl-isothiocyanate (AITC) derived from wasabi, cinnamaldehyde, allicin from garlic and acrolein from cigarette smoke (Bandell *et al.*, 2004; Macpherson *et al.*, 2005; Birrell *et al.*, 2009). TRPA1 can also be activated by endogenous ligands that are produced in

pathological states, including lipids like 4-hydroxy-2-nonenal and 4-oxo-nonenal (Trevisani *et al.*, 2007; Zherebitskaya *et al.*, 2009). Such endogenous ligands can be by-products of oxidative stress, and by their nature oxidants will be able to react with TRPA1. TRPA1 is therefore also well known as an oxidant sensor (Bessac *et al.*, 2008; Sawada *et al.*, 2008a). Oxidant sensitivity is also a mechanism by which TRPA1 can be activated by environmental pollutants such as transition metals and ozone (Gu & Lin, 2010; Taylor-Clark & Undem, 2010).

Activation of TRPA1 causes cough in both humans and guinea pigs (Birrell *et al.*, 2009; Brozmanova *et al.*, 2012). In animal models, elements from cigarette smoke can activate TRPA1 (Andrè *et al.*, 2008). In terms of associations with disease, TRPA1 has been shown to play a role in asthma, genetically modified mice lacking functional TRPA1 demonstrated reduced leukocyte infiltration into the airways after allergen challenge and also significantly reduced airway hyperreactivity in response to contractile stimuli (Caceres *et al.*, 2009). The late asthmatic response in ovalbumin sensitized animals is also blocked by selective TRPA1 antagonists (Raemdonck *et al.*, 2012). TRPA1 may therefore drive asthmatic airway inflammation. Additionally, during inflammatory events endogenous mediators of TRPA1 are produced that can also activate the channel (Taylor-Clark *et al.*, 2007; Grace *et al.*, 2012; Chen & Hackos, 2015).

1.5 Airway sensory nerves and DEP

An increasing number of individuals are being exposed to urban air pollution. There are now well established associations between exposure to DEP and adverse respiratory effects (Sydbom *et al.*, 2001; Kagawa, 2002; Pronk *et al.*, 2009; Weiss *et al.*, 2011). Efforts are being made to understand the cellular mechanisms that DEP can cause such effects, however comparatively few studies have directly investigated how airway sensory nerves activation may play a role.

When comparing the respiratory reflexes that are downstream of airway sensory nerve activation, there are clear overlaps with the respiratory symptoms that are associated with DEP exposure such as cough, bronchoconstriction and inflammation (Nicolai *et al.*, 2003; Belvisi, 2003a; Groneberg-Kloft *et al.*, 2006; McCreanor *et al.*, 2007; Nasra & Belvisi, 2009). For healthy individuals, the severity of respiratory symptoms triggered by real world DEP exposure are relatively mild. However individuals who have underlying respiratory disease such as asthma or COPD are more acutely affected by DEP exposure (Nordenhäll *et al.*, 2001; Pandya *et al.*, 2002; Hart *et al.*, 2012; Sinharay *et al.*, 2013). Airway sensory nerve function has been shown to have a role in these disease states, with hypersensitivity to stimuli being

implicated as the reason for symptoms such as excessive cough being reported in patients (Morice, 2010; Belvisi *et al.*, 2016).

On a more conceptual level, the explicit function of airway sensory nerves is to protect the airways by responding to hazardous stimuli. This occurs through the activation of TRPs expressed on their surface acting as environmental sensors. Other air pollutants such as ozone have been shown to activate airway sensory nerves (Taylor-Clark & Undem, 2010), and there are reports of PM being able to activate TRP channels. TRPV1 is expressed in pulmonary epithelial cells, and *in vitro* and *in vivo* exposure to PM stimulates immunomodulatory cytokines (IL-6, IL-8 and TNF-α) in these cells (Veronesi, 1999). Residual coal fly ash is an industrial pollutant that can activate both human bronchial epithelial cells and nociceptive sensory neurons via TRPV1 pathways (Oortgiesen *et al.*, 2000). Apoptotic events that occur in response to exposure to ash-based environmental PM in murine sensory neurons has been shown to be due to TRPV1 mediated Ca^{2+} influx (Agopyan *et al.*, 2003).

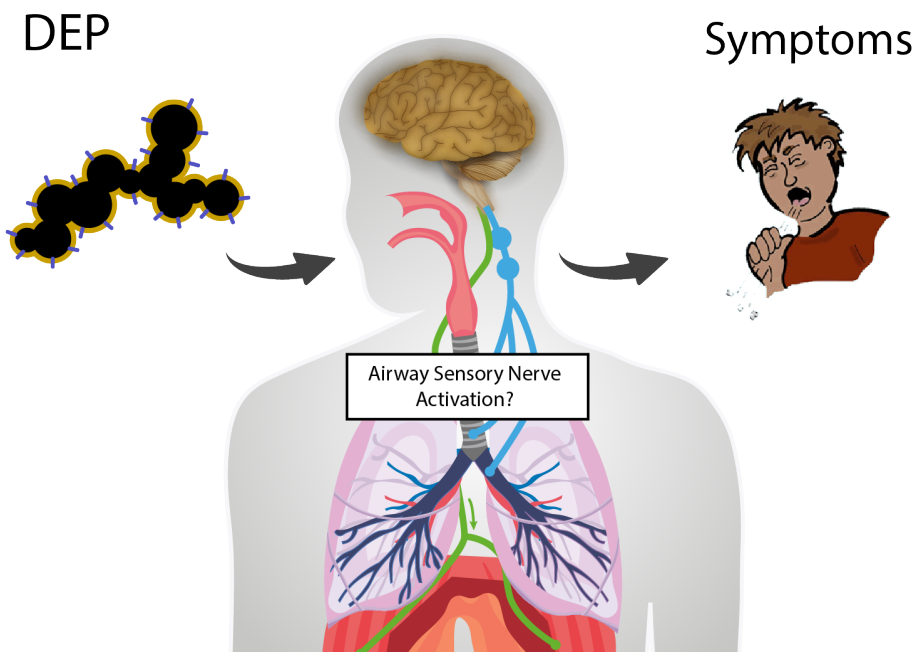


Figure 1.13: Can diesel exhaust particles activate airway sensory nerves to cause respiratory symptoms?

Although there is clear evidence that DEP exposure is associated with respiratory symptoms, the cellular mechanisms by which this process occurs it is not yet completely understood. Activation of airway sensory nerves is a novel mechanism that may in part explain the effects of DEP exposure, as many of the respiratory reflexes that occur due to airway sensory nerve activation are similar to those reported by epidemiological studies into the adverse effects of DEP.

It therefore seems plausible that airway sensory nerves can be activated by DEP and that this is the potential underlying mechanism that drives the observed respiratory symptoms associated with urban air pollution. In rats, a bilateral vagotomy reduced DEP induced airway inflammation as measured by increased neutrophils in bronchial alveolar lavage (BAL) fluid (McQueen *et al.*, 2007). The effect of DEP on TRP channels has only been investigated in a limited number of studies. Intratracheal administration of DEP into the rat lung produced oedema that was significantly reduced by pre-treatment with capsaicin, a technique that desensitises TRPV1 and depletes neuropeptide release (Teles *et al.*, 2009). In human epithelial cells, DEP evoked inward Ca^{2+} via a TRPV4 mediated mechanism and promotes the secretion of MMP-1 which plays a role in inflammatory remodelling of the lung (Li *et al.*, 2011) (Deering-Rice *et al.*, 2011) found that DEP could activate TRPA1 and induce inward Ca^{2+} currents in mouse dorsal root ganglia neurons. The majority of this activity could be preserved in the ethanol extractions of DEP.

While activation of these TRP channels has been shown to initiate respiratory reflexes, these previously studies have not specifically investigated whether DEP can initiate such mechanisms in airway sensory nerves. If this were to be true, it would provide a novel neuronal mechanism that linked DEP exposure to the various respiratory symptoms that are observed (Fig 1.13). Knowledge of the cellular mechanisms by which DEP causes adverse health effects will aide in the understanding of how to prevent the harm caused by air pollution and potentially drive policy change to ensure that individuals do not suffer respiratory distress simply be breathing the air around them.

1.6 Thesis plan

Hypothesis: DEP can activate TRP channels expressed on airway sensory nerves.

To investigate this hypothesis, the aims of this thesis are to:

- Determine whether DEP can activate airway sensory nerves using in vivo and in vitro systems.

- Investigate which components of DEP are responsible for the activation of airway sensory nerves

- Identify whether specific TRP channels are involved in the activation of airway sensory nerves by DEP using selective pharmacological tools and genetically modified animals.

- Investigate the potential mechanisms and signalling pathways underlying the activation of airway sensory nerves by DEP.

These aims will be achieved by:

- Physicochemical characterization of DEP using a range of analytical techniques.
- Using *in vivo* electrophysiology to measure airway sensory nerve fibre firing in response to DEP.
- Using *in vitro* electrophysiology to determine the effects of DEP and specific pharmacological tools on an isolated vagus nerve preparation.
- Additionally, key experiments will be replicated in human tissue surplus to translate requirements, enabling translation of findings elucidated from animal tissue

2 Chapter Two: Methodology

This chapter will detail the general methodology of the techniques used for my thesis. As well as outlining experimental methodology, this chapter will also detail the advantages and principles behind the techniques. Detailed methodology including experimental protocols and statistical analysis can be found in the methods section for each chapter.

2.1 DEP preparation

2.1.1 Particle Dispersion

Due to the hydrophobic nature of DEP, proper dispersion within aqueous solutions required sonication. DEP was prepared in either Krebs solution (in mM: NaCl 118; KCl 5.9; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25.5; glucose 5.6) for *in vitro* studies, or phosphate buffered saline (PBS) for *in vivo* studies at a stock concentration of 0.5mg/ml in a glass vial. The solution was then briefly vortexed before placed in a sonicating water bath (FB 11021 - Fisherbrand) for 5 minutes. The glass vial was then inverted several times and then placed back into the sonicating water bath for an additional 5 minutes. This stock DEP solution was then diluted in appropriate physiological solutions to working concentrations for subsequent experiments.

2.1.2 Soxhlet Extraction

Soxhlet extraction was utilized to prepare an organic extractions of DEP. This technique allows for the extraction of chemicals from a solid sample into a small volume of solvent, leaving insoluble components behind. The sample is suspended within a paper thimble held in a main reservoir in the middle of the apparatus, with a solvent reservoir at the base and a water condenser at the top. As the solvent is heated, it passes through a connector to the condensing water jacket above the thimble. As the solvent cools, it falls down into the thimble and extracts chemicals from the solid sample. As the volume of the solvent in the thimble increases, it eventually triggers a siphoning system and flushes down back to the reservoir. Thus a small amount of solvent can be constantly recycled as the solvent continues to be heated, allowing a complete chemical extraction to occur.

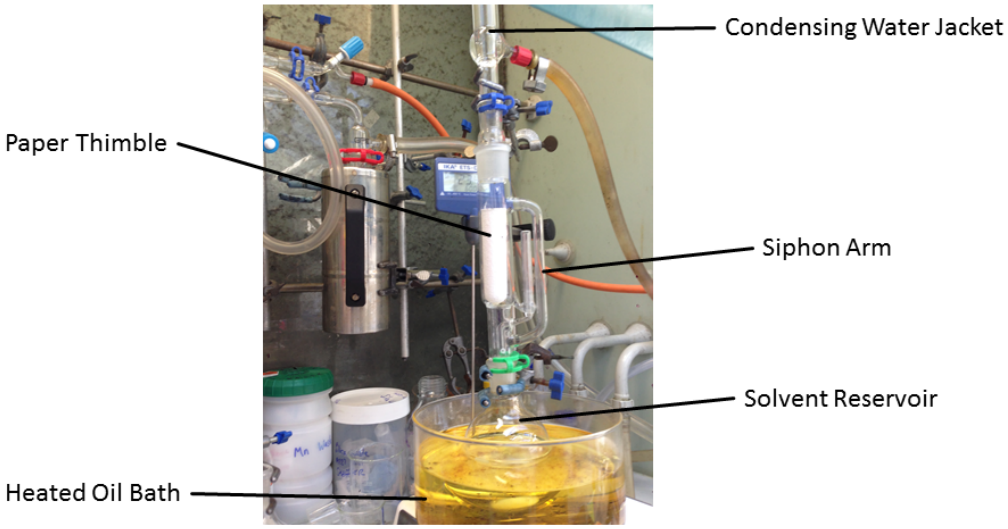


Figure 2.1: Soxhlet extractor apparatus.

Soxhlet extraction was utilized to extract soluble organic chemicals from insoluble components of DEP using a small volume of DCM as a solvent.

1 g of DEP was loaded into a paper filter and extracted overnight with dichloromethane (DCM) using soxhlet extraction equipment at 40°C. The extract was then evaporated by rotary distillation, and reconstituted in dimethyl sulfoxide (DMSO) up to a volume of 10ml. This resulted in an 'equivalent concentration' of 0.1mg/ml of organic material in DMSO. The resulting self-made organic extract of DEP (org-DEP) was centrifuged at 10,000g for 20 minutes to remove remaining particulates and supernatant carefully removed. DEP-OE was then diluted in DMSO and stored at -80°C until needed. The remaining particles left behind in the paper filter were heated gently overnight to completely evaporate any remaining DCM, and were then kept as cleaned particles (par-DEP).

2.2 Physicochemical characterization

One of the major challenges when studying the biological effects of DEP is its highly complex and heterogeneous composition. As such, it is important to determine the physicochemical properties of DEP so that physiological results can be put into proper context.

2.2.1 Dynamic Light Scattering

Dynamic light scattering (DLS) is a technique used to measure the size of particles in solution. DLS involves the measurement of the Brownian motion of particles from which average

particle size is calculated based on the principle that larger particles will move more slowly through a solution than smaller ones.

One limitation of DLS as a measurement technique is that for samples that contain a heterogeneous size population the presence of larger particles will mask the smaller particles, even if the larger particles are present only as a small percentage of the total particle number.

DLS measurements were carried out using a ZetaSizer Nano ZS (Malvern, UK). Samples were warmed to 37°C and left to equilibrate for 60 seconds prior to DLS measurements, which were carried out at a 173° backscatter angle and in triplicate for each sample. Measurement run durations were automatically calculated on a per sample basis.

2.2.2 Electron Microscopy

Given that the size of individual DEP are measured on the nanometre scale, electron microscopy is an ideal technique to visualize the structure and morphology of samples. Conventional optical microscopy has an upper limit for useful magnification due to the diffusion of photons, making resolution of objects smaller than 200 nm difficult. Unlike conventional optical microscopy, electron microscopes use a beam of accelerated electrons to strike a sample. With electrons possessing a much shorter wavelength than photons, EM allows for much higher spatial resolution than optical microscopy, resulting in structures at the atomic level to be identified.

Transmission electron microscopy (TEM) utilizes a high energy electron beam (typically 40 keV to 400 keV) targeted at thin sections of a sample and the interactions between the electrons and atoms of the sample can be detected by a charge-coupled device. One limitation of TEM is that the sample must also be analysed in a vacuum, as the presence of molecules within normal air would interfere with the cohesiveness of the electron beam. During the generation of a vacuum, water is removed from a sample. However, to make the imaging of DEP to be as relevant as possible to the *in vitro* experiments, DEP were prepared physiological solutions. These would have produced salt deposits upon desiccation that would have made conventional TEM impossible.

2.2.2.1 Cryogenic Electron Microscopy

Cryogenic electron microscopy (cryo-EM) involves the rapid freezing of samples before transferring them into a high vacuum TEM for imaging and chemical analysis. By rapidly freezing samples to liquid ethane temperatures (-150˚C), vitrification of water can occur whereby ice crystals to not have time to form and thus the particles can be frozen in their native state, preserving their dispersion and agglomeration.

The cryogenic preparation was carried out using a Leica GP automatic plunge freezer (Leica Microsystems, Milton Keynes). DEP (1µg/ml), were dropped onto a grid and frozen by rapidly plunging into liquid ethane. These were transferred in their frozen state into a cryo-rod (Gatan 914) and then into the electron microscope. The electron microscopy was carried out on a Jeol 2100F equipped with a Schotky Field Emission Gun and operated at 200kV.

2.2.2.2 Energy Dispersive X-Ray Analysis

As well as providing morphological information about a specimen, TEM also allows for compositional information to be calculated by using appropriate detectors to analyse signals given off interactions of the primary electron beam with the specimen.

The underlying principal of transmission electron microscopy energy dispersive X-ray (TEM-EDX) spectroscopy is to strike a sample with a high energy electron beam. This will excite electrons found within the inner shells of atoms of the sample. The excited electron will then leave the inner shell and ascend to an outer shell. To compensate for the missing electron in the inner shell, an electron from the outer shells then drops down to return the atom to a stable state. However, to do so requires the shedding of energy from the electron through the emission of X-Rays. The specific X-ray energy patterns emitted are dependent on the unique atomic structure of each element, thus using an X-ray detector allows for atomic compositional information can be obtained about the sample.

DEP samples were dispersed by sonication in ethanol and then pipetted onto a grid at room temperature. TEM-DEX analysis was performed on an FEI Titan 80–300 (S)TEM operated at 300 kV, fitted with a Cs (image) corrector and SiLi EDX spectrometer (EDAX, Leicester UK). EDX was carried out by condensing the electron beam onto the region of interest and acquiring spectra for 180 seconds.

2.2.3 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is a commonly used technique to identify the physicochemical properties of a material through heating in a controlled environment. By closely monitoring the mass change of a sample as the temperature is increased, information can be gathered on the sample's composition. TGA is particularly suited to identifying the purity of carbonaceous material and inorganic residue content. Due to the combustion profiles of the different components, carbonaceous content can be calculated as well as inorganic residue content (such as trace metal species).

DEP samples were heated using a Perkin Elmer Pyris 1 (PerkinElmer Inc., Beaconsfield, UK). 1.8 ± 0.2 mg of DEP was heated to 100 °C, under air flow (flow rate 10 mL/min), and holding isothermally for 30 minutes to remove residual water and/or solvent; the temperature was then increased from 110 °C to 850 °C at a constant ramping rate of 10 °C/min under air flow (10 mL/min).

2.3 Animal tissue

Male Dunkin Hartley guinea pigs and wild type (WT) mice on a C57BL/6 mice were purchased from Harlan (Bicester, UK). All animals were housed in the Central Biomedical Services animal housing facility at Imperial College London. Animals were kept in a climate controlled room (kept at 21 °C) with food and water freely available for at least 1 week before commencing experiments. All experiments were performed following approval by the Imperial College London Ethics Review Committee, and in accordance with the U.K. Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act of 1986 under the Project License Number 70/8845.

2.3.1 Generation of genetic knock out mice

Although the use of pharmacological tools allows for investigations into the cellular signalling mechanisms, in cases where the tools are not available or lack the required specificity the genetic deletion of the targets of interest.

2.3.1.1 Mouse colony breeding

All mice were bred on a C57BL/6 background. Homozygous breeding pairs of genetically modified mice with disruptions to TRPV1 ($TRPV1^{-/-}$) and TRPA1 ($TRPA1^{-/-}$) genes were purchased from Jackson Laboratories (USA). Homozygous breeding pairs of mice with disruptions to the TRPV4 gene ($TRPV4^{-/-}$) were obtained from Riken Bioresource Centre (Tsukuba, Japan).

All KO mice were viable and fertile; breeding colonies were maintained within the Central Biomedical Services animal housing facility at Imperial College London. Animals were kept in a climate controlled room (kept at 21 °C) with food and water freely. Additionally, colonies of WT C57BL/6 mice were bred at the same times to be used as controls to compare the effects of genetic modified mice.

2.3.1.2 Genotyping

Tail tips of mice were taken and DNA extraction performed using a DNA extraction kit ('Extracta DNA prep for PCR', Quanta Biosciences, Gaithesberg, USA). Tail tip sections (approximately 4mm in length) were placed into a 200 µl Eppendorf tube and 75 µl of extraction buffer was added. After heating at 95°C for 30 minutes, the tail tips were cooled to room temperature and 75 µl of stabilization buffer added. DNA concentrations were analysed using spectrophotometry at wavelengths A260/A280 using GeneQuant RNA/DNA quantifier (Amersham Pharmacia, UK). DNA concentrations were then adjusted to 10ng/ml using nuclease free water.

Polymerase Chain Reaction (PCR) amplification was then used to amplify the DNA sequences of interest. A reaction mixture containing 50ng of extracted DNA (5 µl), reaction buffer (5 µl), 0.2mM dNTPs (1 µl), 2mM $MgCl_2$, (2 µl), 1.25U Taq polymerase (0.2 µl), 10 pmol forward and reverse primers of the target of interest (1 µl) and the volume then made up to 25 µl using nuclease free water. PCR reagents were purchased from Promega, UK and primers purchased from Invitrogen, UK.

The reaction mixture was then heated to 94°C and 40 repeated cycles of denaturing, annealing and extension were carried out. The exact temperatures and timings used for each were dependent on the sequence of primers used and the expected length of the DNA sequence being amplified. Following 40 repeated cycles, the reaction mixtures were then held at 72°C for ten minutes, then the PCR reaction stopped by cooling the samples to 4°C for 5 minutes. PCR products were then run on a 2% agarose gel at 80V for 1 hour in a Tris/Borate/EDTA (TBE) buffer containing 0.05 µl/ml Safeview (NBS Biologicals Ltd.

Huntingdon, UK) with an appropriate reference DNA ladder. The gel was then visualized under ultraviolet light and photographed to confirm the presence of PCR products and their size.

2.3.1.3 Limitations

The genetic modifications made to mice for research purposes can lead to problems with fertility, development and health of the animal. However, the TRP KO mice used for my thesis research do not carry such deficiencies. Genetic KO of target genes may also not result in the physiological loss of function expected. Redundant signalling pathways exist within the cell that may allow for the up regulation of compensatory genes so that the functional consequence of gene deletion is minimal. Finally, variations between the mouse and the human genome mean that there may be differences in the role that specific genes play across species.

2.4 Human tissue

The use of animal models allows for a large number of experiments to be performed. However, while many biological pathways are preserved between species, inherent genetic differences mean it is also critical to ensure that the results obtained can also be translated to human biology. Due to the high cost and relative scarcity of human tissue, only key experiments will be selected to be replicated.

2.4.1 Sourcing

Human vagus tissue was obtained from donor tissue that was unsuitable for transplant and surplus to requirements. Whole en bloc lungs with the vague nerve still attached were purchased from the International Institute for the Advancement of Medicine (IIAM, NJ, USA) and received on ice in stored in transplant solution (Perfadex, University of Wisconsin solution or Histidine-tryptophan-ketoglutarate) no more than 36 hours from cross clamp. In all cases the tissue was approved for use in scientific research and ethical approval was obtained from the Royal Brompton & Harefield Trust.

2.4.2 Selection criteria

The selection criteria for donors was as follows:

Age	18+ accepted.
Respiratory Diseases	Asthma, COPD accepted
Cancer	Past/Present accepted except lung cancer
Blood serology	Only Cytomegalovirus and Epstein–Barr virus accepted.
Infections	Reject for Methicillin-resistant Staphylococcus aureus (MRSA), bacterial/viral meningitis, sepsis.
Ventilation Time	Less than 14 days

2.5 Determination of gene expression via mRNA measurement

Real time PCR analysis tissue isolated from guinea pig vagal ganglia was carried out to confirm that the TRP genes of interest are present in sensory nerves. The jugular and nodose ganglia house the neuronal cell bodies that make up the vagus nerve, including those that innervate the airways. Detecting messenger ribonucleic acid (mRNA) of the TRP channels of interest would indicate that these channels are also present along the vagus nerve.

2.5.1 RNA Extraction of whole ganglia

Guinea pigs were sacrificed using an overdose of sodium pentobarbitone (200 mg/kg intraperitoneal injection). Nodose and jugular ganglia were dissected, cleaned of connective tissue and placed into sterilised 2ml eppendorf tubes. TriReagent (1ml, Sigma) was then added to each eppendorf and the samples were centrifuged (15,000g for 15mins at 4oC) to remove any insoluble material such as cell membranes. The supernatant (containing RNA, DNA and protein) was then transferred into a new eppendorf and left at room temperature (RT) for 5 minutes. Next, 0.2ml of chloroform was added and the eppendorf agitated to facilitate mixing before being left at RT for 15mins The samples were then centrifuged (15,000g for 15min at 4°C). The samples separated into three distinct phases in the eppendorf, the organic phase (protein), interphase (DNA) and aqueous phase (RNA). The RNA phase was recovered by aspiration, placed into a 1.5ml eppendorf, mixed with 1:10 isopropanol (Sigma) and left at RT for 5 minutes following which the sample was centrifuged (12,000 x g, 10min, 4°C). Additional isopropanol was added give a final volume of 0.5ml, then the sample left to stand for 5mins at RT. Following subsequent centrifugation (12,000 x g, 10min, 4°C), a precipitate of RNA formed and the samples were then kept on ice. The supernatant was removed and sample washed with 1ml of 70% ethanol, vortexed and centrifuged (12,000 x g

for 5 minutes, 4˚C). The supernatant was removed and the sample dried for 5 minutes, following which the RNA pellets were dissolved in 50µl nuclease free water.

To determine the purity and concentration of the RNA, a small sample was removed and diluted 1:10 using nuclease free water. The diluted RNA was then analysed using A260/A280 spectrophotometry (Birtek Powerwave XS microplate spectrophotometer - BioTek). The concentration of the stock RNA sample was calculated and adjusted to 0.05mg/ml using nuclease free water.

2.5.2 cDNA synthesis

Reverse transcription of RNA samples to synthesise cDNA was carried out using Taqman reverse transcription reagents (Applied Biosystems, UK). A master mix was made up containing 1x Taqman RT buffer (5µl), deoxyNTP mix (500uM/NTP; 10µL), $MgCl_2$ (5.5mM, 11µL), random hexamers (2.5µM; 2.5µL), RNAse inhibitors (0.5U/µL; 1µL) and multiscribe reverse transcriptase (1.25U/µL: 1.25µL) to give a final reaction volume of 50µL. Using a thermal cycler (model 480; Perkin Elmer, Boston, USA), samples were then incubated for 10 mins at 25°C, 30 mins at 48°C and 2 minutes at 95°C. Resulting cDNA samples were then frozen at -80°C until required to be used for RT-PCR.

2.5.3 Ganglia tissue TaqMan RT-PCR

Taqman RT-PCR probes contain a fluorescent reporter dye. Successive copies of cDNA that are transcribed causes an increase in fluorescence. RT-PCR amplification was carried out using an ABI PRISM 7000 Taqman machine (Applied Biosystems). A reaction mix (25µL) was prepared containing 2x Taqman universal master mix containing Taq DNA polymerase (12.5µL), sample cDNA (10ng), Assay On Demand reagent containing specific primers and probes (1.5µL) and 18s internal control (1.5µL) (All regents from Applied Biosystems). The amplification protocol was as follows: 1 cycle at 50°C for 2mins, 1 cycle at 95°C for 10mins, 40 cycles at 95°C for 15 seconds and finally the sample is kept for 1 minute at 60°C.

Samples were analysed using Sequence Detection Systems software (v 1.2.1, Applied Biosystems, UK). 18s is used as a control, as this gene is present in all eukaryotic cells. The control signal can be compared to the target cDNA probe to give relative expression of the gene of interest. A manually threshold value was set on the amplification plot on the geometric (linear) phase of amplification as per the manufacturer's instructions. The Threshold Cycle (C_t)

is the cycle number where fluorescence passes the threshold. A lower C_t value corresponds to a higher concentration of target in the sample, as the threshold value was reached at an earlier cycle. To take into account exponential amplification reactions the data was transformed and presented as $2^{-\Delta Ct}$. This allows different mRNA levels to be compared relative to the expression of the endogenous 18S control within each sample.

2.5.4 Single Cell RT-PCR

As a more refined technique, single cell RT-PCR allows for the detection of RNA targets of interest in specific individual cells, rather than whole tissue samples. Experiments were performed with the assistance of Dr. Michael Wortley. Male Dunkin Hartley guinea pigs were intranasally dosed with a retrograde tracer dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). 14 days later, the nodose and jugular ganglia were enzymatically digested and neurons were plated in glass bottom fluorodishes at 37˚C in a humidified atmosphere (5% CO_2/95% O_2) overnight.

Airway terminating (DiI positive stained) neurons were identified using a confocal light microscope (Leica inverted confocal microscope with a Radiance2000 system). A pulled-glass micropipette (tip ID, 50-70 µm; OD, 2 mm; FIVEphoton Biochemicals) was manoeuvred into position above the selected neurons using a micromanipulator (Three-axis Water Hydraulic Micromanipulator MHW3, Narishige). Neurons were then harvested using manual suction into the end of the micropipette, after which the the tip of the pipette broken off into a sterile microreaction tube containing 1 µL of RNaseOUT (Life Technologies) and placed on ice. Cells were processed by using the Superscript III Direct cDNA Synthesis Kit (Life Technologies) and Random Hexamers (Applied Biosystems) per the manufacturer's instructions and as described by (Lieu *et al.*, 2011).

Some samples were removed immediately before addition of Superscript III reverse transcriptase to be used as −reverse transcriptase (RT) controls. Sample cDNA and −RT controls were stored at −20°C until PCR analysis. The reaction mixture for PCR contained 0.5 U of HotStarTaq DNA Polymerase, 2.5 mmol/L $MgCl_2$, and PCR buffer (HotStarTaq Polymerase; Qiagen, Hilden, Germany), 200 µmol/L dNTP and custom-synthesized intron-spanning primers (0.5 µmol/L each of forward/reverse primer). The PCR reaction conditions were 95°C for 15 minutes and then 50 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. PCR products were then run on a 2% agarose gel at 80V for 1 hour in a TBE buffer containing 0.05 µl/ml Safeview

(NBS Biologicals Ltd. Huntingdon, UK) with an appropriate reference DNA ladder. The gel was then visualized under UV light and photographed to confirm the presence of PCR products and their size.

2.5.5 Limitations

While gene expression is a useful tool to investigate whether any targets of interest are expressed in the RNA of the cell, it must be remembered that expression on the gene level does not necessarily translate into functional proteins.

To determine whether functional proteins are present, other analytical techniques such as western blot must be used. Reliable results from western blot experiments requires research grade antibodies that are specific for their targets. Unfortunately, such high quality tools are not yet available for TRP channel research. Therefore at present, RNA expression is one of the most practical techniques to identify whether it is likely that TRP channels are present.

2.6 *In vivo* single fibre experiments

The activity of airway sensory afferent nerves can be measured *in vivo* using extracellular electrophysiology techniques. Action potential firing can be measured in response to tussive stimuli introduced into the airways, and thus this technique can be used to demonstrate the physiological effects of the inhalation of substances in a live animal. Additionally, these recordings are made from individual airway sensory nerve fibres which allows for responses to be profiled across the various nerve fibre subtypes present. The profile of sensory nerve fibre activation is useful in identifying the underlying mechanisms by which substances affect the lung.

2.6.1 Animal surgery

Guinea pigs were anaesthetized with urethane (1.5 g/kg, i.p). The trachea was cannulated and the animal mechanically ventilated (Ugo Basile small animal ventilator, tidal volume 10 ml/kg, 50-60 breaths per min of laboratory air). Body temperature was monitored by rectal thermometer and maintained at 37°C using a heated blanket (Harvard Apparatus). The right jugular vein and carotid artery were cannulated to allow for the administration of drugs and to monitor arterial blood pressure (Gould P23XL transducer) respectively. The animal was then paralyzed by vercuronium bromide (initial dose 0.1 mg/kg i.p, followed by 0.05 mg/kg every

20 minutes to maintain paralysis). The level of anaesthesia was assessed by monitoring heart rate and blood pressure responses to noxious stimuli throughout the experiment. The two vagus nerves were then cut centrally. The skin and muscle at the neck were then attached to a metal ring supported above the animal to create a well, which was then filled with light mineral oil.

2.6.2 Electrophysiological recordings

The left vagus nerve was carefully cleaned, dissected and teased down to a single nerve fibre by hand under a microscope and placed onto a small black Perspex plate. The single nerve fibre was then put into contact with a bipolar Teflon-coated platinum electrode. A second electrode was placed into contact with fascia to provide a reference signal. Measurements of action potential firing were recorded though a pre-amp headstage (Digitimer NL100K). The signal was amplified (x5000 Digitimer NL104), filtered (low pass – 30 Hz, high pass 8.5 kHz, Digitimer NL125) and passed through a Humbug noise reducer (AutoMate Scientific) before sampling and recording. Signals were sampled at 50 Hz and recorded using Spike 2 software data acquisition system via a CED Micro1401 interface.

2.6.3 Nerve Fibre Subtype Identification

During the course of the experiment the nerve fibre subtype was identified using a set of criteria. Nerve fibres were confirmed to innervate the airways through various selection criteria, including subtypes identified by patterns of spontaneous activity, responses to hyperinflation or hyperdeflation and application of the tussive stimuli such as capsaicin (100μM) or citric acid by aerosol administration. Aerosols were generated by an Aerogen nebulizer (Buxo Nebulizer Control – 5) connected to the ventilator. If the fibre has minimal spontaneous activity, it does not respond to lung inflation and it responds to capsaicin, then it is identified as a C fibre. Conversely, if the nerve fibre displays consistent spontaneous activity, it clearly responds to lung inflation and it responds to citric acid (0.3M) then it is assumed to be an A-δ fibre. This rough classification is made during the early stages of the experiment, and at the end of the experiment nerve subtypes are confirmed using conduction velocity tests. Slowly conducting afferents were identified as non-myelinated C-fibres and quickly conducting myelinated Aδ-fibres. This was achieved by stimulating the vagus nerve close to the thorax using a supra threshold voltage (Grass stimulator, 0.5 ms, 1Hz). The corresponding action potential was recorded in the single nerve fibre under observation, and the time between the

initial stimuli and the resulting action potential was calculated using the Spike 2 software and used with the measured distance between the two electrodes to calculate the conduction velocity. All animals were culled at the end of experiments with an overdose of pentobarbitone.

2.6.4 Agonist experimental protocol

The following is a general experimental protocol. Please consult the relevant later chapters for specific timings and drug concentrations used.

Following the identification of a suitable airway fibre, baseline activity was recorded over two minutes. The vehicle for the agonist of interest was then administered to the lungs during which potential action potential activity was recorded and intratracheal pressure and blood pressure were monitored. A recovery period of 10-20 minutes then followed to allow for changes to return to baseline. Then, capsaicin (100μM, 15s) was administered by aerosol and subsequent action potential activity was recorded. A recovery period of 10-20 minutes then follows to allow for changes to return to baseline. Then, citric acid (0.3M, 1 min) was administered by aerosol and the same recordings made.

Following another recovery period of 10-20 minutes, the agonist of interest was administered for 1 minutes and subsequent action potential activity was recorded. After changes returned to baseline following a final 10-20 minute recover period, the viability of the nerve was confirmed using a final stimulation using either aerosolized capsaicin (100μM) for C-fibres or citric acid (0.3M) for A-δ fibres. At the end of the experiment, conduction velocity tests were used to confirm the nerve fibre subtype (see 2.6.3 – Nerve Fibre Subtype Identification).

Data was expressed as mean ± SEM and analysed using the paired students t-test, comparing responses immediately prior to stimuli administration as baseline values compared to the responses recorded in the two minute period following stimulus administration.

2.6.5 Antagonist experimental protocol

The following is a general experimental protocol for identifying the effects of antagonists on responses induced by agonists on C or A-δ airway fibres. For specific timings and drug concentrations used, please consult the later chapters.

Following the identification of a suitable airway fibre, baseline activity was recorded over two minutes. Then, capsaicin (100μM) was administered by aerosol and subsequent action potential activity was recorded, as well as changes in intratracheal pressure and blood pressure. A recovery period of 10-20 minutes then follows to allow for changes to return to baseline. Then, citric acid (0.3M) was administered by aerosol and the same recordings made.

After responses had returned to baseline, two repeatable control responses to the agonist of interest were obtained. Following a return to baseline, the antagonist of interest is then administered to the animal via intraperitoneal injection for 1 hour. After antagonist treatment, the agonist is administered to the animal in the presence of the antagonist and responses recorded.

2.6.6 Limitations

Single fibre recordings are unable to directly measure the isolated effects of single receptors. Instead, inferences are made that action potentials are likely to be generated by the activity of a single sensory receptor.

One major limitation of single fibre recordings is the length of time each experiment takes, as a single data point will take several hours to obtain even with a highly skilled experimenter. Additionally, each single data point will use one animal at a time, increasing the number of total animals used and experimental costs compared to other techniques that can obtain multiple data points from a single animal. Due to this difficulty in the generation of large data sets, single fibre recordings will be used to validate the key findings from higher throughput techniques.

2.7 *In vitro* isolated vagus nerve preparation

Exclusively in vivo techniques to investigate pharmacological mechanism behind DEP activation of airway sensory nerves would be prohibitively expensive and time consuming, due to the number of animals that would need to be used. In contrast, the isolated vagus nerve preparation is an ideal technique as multiple experiments using different combinations of test solutions can be carried out using the tissue of a single animal and is therefore a higher throughput technique. If an experiment is designed to investigate whether an agonist can directly activate airway sensory nerves then the *in vitro* isolated vagus nerve preparation has additional advantages over the *in vivo* preparations as the nerve is not also subjected to

additional factors such as mucus production, bronchoconstriction or other airway inflammatory events that can make interpretations of the findings difficult.

Another important advantage is that nerve tissue from different species can be used. This allows experiments using animal tissues to be paralleled in human tissue, to determine whether the findings obtained are translational. Previous studies have shown that results from guinea pig and human isolated vagus nerve experiments to be comparable. Additionally, this allows the use of genetic knock-out mice tissue to be used to confirm mechanisms identified using pharmacological tools.

2.7.1 Tissue dissection: Human

Whole lungs that were unsuitable for transplant were purchased from IIAM and received on ice in transplant solution (Perfadex, University of Wisconsin solution or Histidine-tryptophan-ketoglutarate) no more than 36 hours from cross clamp. Once received, the lungs were immediately placed into modified Krebs solution and bubbled with 95% O_2/5% CO_2. Vagus nerves running parallel to the trachea and bronchi were dissected, before being carefully cleaned and desheathed under a dissecting microscope. The cleaned nerves were then cut to segments approximately 20mm in length. Throughout the experiment, care was taken so that the nerves remained in oxygenated Krebs and were not physically damaged.

2.7.2 Tissue dissection: Animal

Guinea pigs and mice were sacrificed by an overdose of pentobarbitone (200mg/kg. i.p). For example, for a guinea pig with a typical weight of 350g, 1ml of pentobarbitone was injected by intraperitoneal injection, with the volume equally distributed on either side of the animal. Death was confirmed by cessation of heartbeat (as determined by palpation), followed by exsanguination by severing of the aorta.

A midline incision at the neck was made to expose the trachea and thorax, then the muscle and connective tissue removed to expose the vagus nerves. Both vagus nerves were cut caudally to the nodose ganglion and proximally to the heart, these segments were then placed into modified Krebs solution and bubbled with 95% O_2/5% CO_2. The nerves were then carefully cleaned of additional connective tissue and desheathed. The cleaned nerves were then cut to

segments approximately 20mm in length. Throughout the experiment, care was taken so that the nerves remained in oxygenated Krebs and were not physically damaged.

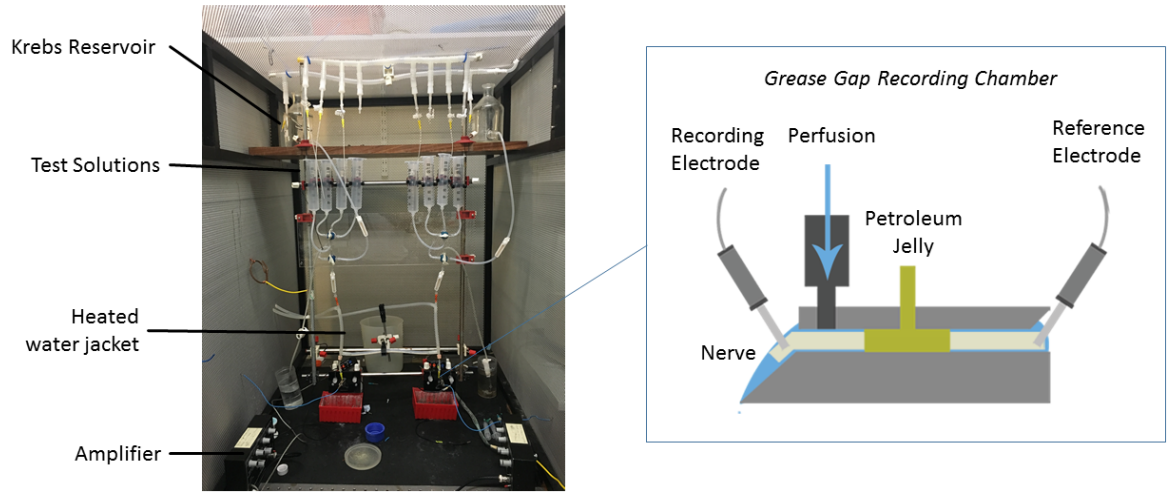


Figure 2.2: *In vitro* isolated vagus nerve set up.

Sections of the isolated vagus nerve were mounted into a custom made grease gap recording chamber and electrically and chemically isolated using petroleum jelly. The nerve was superfused at a rate of 2ml/min with either Krebs or test solutions and kept at 37°C using a heated water jacket. Nerve depolarisation induced by test solution was measured by electrodes and amplified using DAM50 differential amplifiers and recorded using a chart recorder.

2.7.3 Isolated vagus nerve electrophysiological recordings

Segments of the vagus nerve were mounted in a grease-gap recording chamber by drawing nerves through a narrow channel within a custom made Perspex block. Petroleum jelly was injected through a side port at the middle of the channel, until both ends of the nerve was chemically and electrically isolated from one another. At this point, constant perfusion at the recording end of the nerve was started using oxygenated Krebs (95% O_2/5% CO_2) at a rate of 2ml/min and held isothermally at 37˚C using a thermal jacket and water bath. The other reference end of the nerve was kept in a reservoir of Krebs to serve as a baseline measurement for electrophysiological recordings

Glass rod electrodes (1.5mm in external diameter, 1mm in internal diameter) were attached to Ag/AgCl-pellet half-cell electrode (Mere 2 flexible electrodes, WPI, UK) and completely filled with modified Krebs solution. The end of one glass electrode was then carefully placed in

direct contact with the reference end of the nerve, whilst a second glass electrode was positioned into electrical contact with the reference end of the nerve by being placed into the reference Krebs reservoir.

The placement of the electrodes allows for the compound depolarisation of the nerve to be recorded. When challenged by an agonist superfused onto the recording end of the nerve, activation of the ion channels on the surface of the nerve causes the flow of Na^+ ions to move from the surrounding Krebs solution into the cell. As a result of this ionic movement, excess Cl^- ions in the Krebs solutions react with Ag found within the electrode to form AgCl and free electrons, thus electrically stimulating the circuit. As the reference side of the nerve is not challenge by agonists, there is no ionic flow and thus no change in the electrical potential of the reference end. Thus, depolarisation can be measured as the potential difference between the two ends of the nerve, which is then amplified and recorded on the chart recorder. When the agonist challenge is stopped on the recording end, fresh Krebs is superfused over the nerve and the ion channels revert to a basal state. K^+ ions then leave the cell and cause the Cl^- ions to disassociate from the AgCl pellet. Thus the potential difference is lost between the two ends of the nerve and the nerve repolarizes back to a baseline level.

Changes in the membrane depolarisation were recorded using an extracellular potential differential amplifier (DAM50 Bioamplifier, World Precision Instruments, USA). Nerve depolarizations were filtered at 0.3 kHz, amplified x50, and recorded onto a chart recorder (Lectromed Multi-Trace 2).

2.7.4 Experimental design and protocol

2.7.4.1 Concentration Response

To determine whether an agonist could activate the vagus nerve *in vitro*, the following general non-cumulative concentration response experimental protocol was used. For specific timings and drug concentrations used, please consult the later chapters.

After allowing the vagus nerve to reach a stable baseline, vagal depolarisation was measured in mV in response to single concentrations of agonists administered over 2 minutes. Following the agonist stimulation, the vagus nerve was washed with Krebs until it returned to baseline. The stimulations were then repeated using a range of concentrations, including a vehicle run

to ensure that the vehicle for the agonist had no effect. A submaximal concentration of agonist was then selected for subsequent antagonist studies.

2.7.4.2 Antagonist Studies.

For agonists that do not desensitize after repeated applications, the vagus nerve was allowed to reach a stable baseline before being challenged by an agonist for 2 minutes and the resulting depolarization recorded in mV. Following washing with Krebs a return to baseline, the agonist stimulation was repeated to obtain two comparable control responses. Next the vagus nerve is pretreated with an antagonist for 10 minutes before challenged with the agonist in the presence of the antagonist for another 2 minute stimulation. Nerve viability is then assessed with an end of experiment 2 minute stimulation of the agonist. The depolarization obtained in the presence of the antagonist is then compared to the average of the control responses to determine the effect of the antagonist.

For agonists that irreversibly bind to their targets, the above protocol cannot be used as the agonist cannot be washed off. Instead, nerve viability is assessed by the 2 minute application of a positive control (acrolein – 300μM), and washed back to based line before pre-treatment with 10 minutes of the antagonist of interest. Following antagonist pre-treatment, the nerve is immediately challenged with a 2 minute application of the agonist. Nerve viability is then assessed at the end of the experiment by another application of the positive control. As these experiments do not contain internal controls, nerves treated with antagonists are compared against nerves treated with vehicle controls from the same animal.

2.7.5 Limitations

Whilst the isolated vagus preparation has the benefits of being a higher throughput technique that is amenable to pharmacology, it does still have some limitations to be aware of.

As the recording and reference electrodes measure the difference in membrane potential across the whole of the nerve trunk, the depolarisation responses recorded are a summation of the activity within all the nerves found within the nerve trunk tissue, rather than specifically from airway sensory nerves.

Additionally, this *in vitro* preparation applies agonists directly onto the nerve trunk, whereas *in vivo* agonists that are introduced into the airways will stimulate the nerve termini. As the

necessary cellular signalling components of a nerve are initially constructed within the neuronal cell body before translocation to the nerve termini, there may be differences in receptor expression and intracellular signalling at the nerve trunk compared to the nerve termini.

Despite these limitations, the results obtained using the depolarization of the vagus nerve have been shown to correlate well with parallel studies investigating cough responses *in vivo.* Nevertheless, to ensure the validity of the results obtained in this study, where possible critical experiments will be paralleled using *in vivo* single fibre.

2.8 *In vitro* calcium imaging of isolated airway neurons

2.8.1 Isolated neuron preparation

Male Dunkin Hartley guinea pigs were intranasally dosed with a retrograde tracer dye DiI. 14 days later, the nodose and jugular ganglia were enzymatically digested and neurons cultured overnight at 37˚C in a humidified atmosphere (5% CO_2/95% O_2) on flurodishes coated with poly-D-lysine laminin to aid adherence.

Following incubation, the cells were loaded with Fura-2-AM, a calcium sensitive dye for 40 mins at room temperature and protected from light. The cells were then de-esterified by washing with extracellular solution (ECS – in mM: NaCl 136; KCl 5.4; NaH_2PO_4 0.33; $MgCl_2$ 1; $CaCl_2$ 2.5; Glucose 10; HEPES 10) for 40 mins, then the flurodishes were mounted into a custom built perfusion system and imaged using a Widefield light microscope. Neurons were identified at x20 magnification and the identification of airway innervating neurons was confirmed by the presence of DiI staining (excitation λ=550nM; emission λ=570nM). Intracellular Ca^{2+} levels were then recorded in response to agonists as previously described.

2.8.2 Fluorescence imaging of $[Ca^{2+}]_I$

To determine whether the mechanisms observed in the isolated vagus nerve were translatable to the airway specific neurons, jugular and nodose cells were challenged with antimycin A (20μM), H_2O_2 (10mM) and DEP-OE (1μg/ml and 10μg/ml). Each agonist was tested in a separate experiment, with the following protocol. Neuron viability was first confirmed by application of hyperpotassium solution (K_{50}; 50 mmol/L potassium chloride solution) for 15

seconds at the start of recording; these calcium responses to K_{50} were used to normalise subsequent responses. After the dish was washed with ECS to return the signal to baseline, neurons were exposed to vehicle (0.1% DMSO for antimycin A and DEP-OE, Krebs for H_2O_2) for 240 seconds, before a washing period. The neurons were then exposed to the stimuli of interest for 240 seconds, again followed by a washing period to return to baseline. The neurons were then challenged with acrolein (30μM) for 90 seconds as a TRPA1 positive control, and the dish washed to return to baseline. Finally, the neurons were exposed to K_{50} to confirm viability at the end of the experiment.

2.8.3 Analysis

Results were analysed offline using ImageJ software, and area under the curve calculations carried out using a custom Excel spreadsheet to determine the magnitude of $[Ca^{2+}]_i$ responses. Agonist induced responses were expressed as a percentage of the K_{50} response, to normalise values and allow for comparisons between cells and animals. Results are expressed as mean ± SEM.

3 Chapter Three: Airway Sensory Nerves Activation by DEP

3.1 Rationale

Exposure to diesel exhaust particles (DEP), one of the most common airborne pollutants in urban environments (Shah *et al.*, 2004), has been associated with adverse effects on the respiratory system (Riedl & Diaz-Sanchez, 2005). Epidemiological studies have highlighted that symptoms such as cough, wheeze and bronchoconstriction are associated with DEP exposure (McClellan, 1987; Bernstein, 2012); particularly in those with underlying respiratory conditions such as asthma and COPD. The underlying cellular mechanisms that drive such adverse health effects are not fully understood, so there is a need for further research to be carried out. Existing studies have primarily focused on the ability of DEP to induce inflammatory responses in airway and alveolar epithelial cell (Boland *et al.*, 1999; Amara *et al.*, 2007; Li *et al.*, 2008). The effect of DEP on immune cells such as macrophages has also been investigated (Verheyen *et al.*, 2004; Jantzen *et al.*, 2012).

However, one possibility that has not been investigated is the role that airway sensory nerves may play in the observed responses to DEP. Forming part of the vagus nerve, airway sensory afferents innervate the lungs and, through various receptors expressed on their surface, are capable of detecting noxious stimuli in the airways and initiating protective respiratory reflexes, such as cough (Canning *et al.*, 2006; Nasra & Belvisi, 2009). In this manner, these airway afferents provide critical sensory information on the status of the lungs and the airway, ensuring that harmful temperatures, toxins or other chemicals are avoided or removed from the lungs once encountered.

Given that the symptoms associated with exposure to DEP (Kagawa, 2002; Zhang *et al.*, 2009) are similar to those that occur in response to airway sensory nerve activation (Canning *et al.*, 2006; Nasra & Belvisi, 2009), it is important to investigate whether airway sensory nerves can be activated by DEP. For this thesis the effects of SRM-2975 (DEP), a commercially available DEP generated from a fork lift truck, will be examined. By using a standardized DEP, the effect of variation found in real world roadside samples can be minimized that would otherwise increase the difficulty in data interpretation (Camatini *et al.*, 2010). Furthermore, SRM-2975 has been focus of other DEP studies (Singh *et al.*, 2004; Nemmar *et al.*, 2007; Provoost *et al.*,

2010), therefore the results obtained in this thesis can be placed into wider context to aid in the understanding of the mechanisms involved.

3.1.1 Chapter Hypothesis:

Airway sensory afferents can be activated by DEP.

3.1.2 Aims:

- To determine whether DEP can activate airway sensory afferents *in vivo* by measuring action potential firing in response to an intratracheal dose of DEP in the anaesthetized guinea pig.
- To determine whether DEP can activate the isolated vagus nerve *in vitro*, measuring DEP induced depolarization in both guinea pig and human vagus tissue.
- To confirm that any observed responses are the result of true neuronal depolarization using the sodium channel blocker tetrodrotoxin (TTX).

3.2 Methods

3.2.1 Airway Single Fibre Recordings

3.2.1.1 Surgical preparation

Male Dunkin Hartley guinea pigs (approximately 400g) were prepared for *in vivo* single fibre recordings as described in Chapter 2. Briefly, guinea pigs were anaesthetised with urethane (1.5g/kg i.p.). Guinea pigs were surgically prepared, paralysed with vercuronium bromide (0.1mg/kg i.v.) and mechanically ventilated. Both vagal nerves were cut cervically, then the left vagal nerve was dissected down to a single nerve fibre unit and a platinum recording electrode was used to record action potential firing using Spike 2 data acquisition software (captured using a CED Micro 1401 interface).

3.2.1.2 Intratracheal DEP challenge

Following surgical preparation, the nerve fibre subtype was first identified using criteria as previous described, such as the spontaneous response to pulmonary ventilation and response to chemical stimuli. If a C-Fibre was under investigation, aerosolized capsaicin (100 μM in saline, 15 seconds) was administered to the animal as a positive control. If an Aδ-fibre was under investigation, then citric acid (300mM in saline, 1 min) was administered in a similar manner.

Following identification of a suitable nerve fibre and administration of positive controls, animals were left to stabilize for 30 minutes. Baseline control recordings of nerve fibre activity were then recorded for 2 minutes. Animals were then either challenged with a 200 μl of an intratracheal dose of vehicle (PBS) or DEP (10 μg/ml in PBS) and changes in nerve fibre activity, as measured by action potential firing, were recorded. At the end of the experiment, the conduction velocity of the nerve fibre under investigation was measured to finalise the profiling of the nerve fibre subtype.

3.2.1.3 Analysis

A paired Students t-test was used to compare the number of peak impulses per second recorded during the control period prior to stimulus challenge with the time period immediately following the challenge. The data is expressed as mean +/- S.E.M, with statistical significance set at $P < 0.05$.

3.2.2 Isolated vagal nerve recordings

Vagal tissue was dissected either from male Dunkin-Hartley guinea pigs or human lungs that were unsuitable for donor transplant requirements. Vagus nerve sections were then mounted inside a grease gap recording chamber as described in Chapter 2.

3.2.2.1 DEP concentration response

DEP was prepared by creating a stock solution of 0.5 mg/ml in Krebs solution and sonicated for 10 minutes in a sonicating water bath at 25°C until uniformly dispersed. Serial dilutions were then carried out to create working solutions of DEP in Krebs solution. An appropriate concentration range was selected based on the concentrations used in *in vitro* DEP studies found within the literature (Lehmann *et al.*, 2009; Chaudhuri *et al.*, 2012; Totlandsdal *et al.*, 2015).

To determine whether DEP was able to depolarise the isolated vagus nerve, the following protocol was carried out. Vagal tissue was mounted within the grease gap recording chamber and then challenged with randomized non-cumulative concentrations of DEP (0.1-100 µg/ml) for 2 minutes and the resulting depolarization recorded. The vehicle control (sonicated Krebs) was also tested to ensure that sonication itself had no effect on the nerve. Following DEP challenge, the nerve was washed with Krebs solution until recordings returned to baseline, and the next concentration was applied.

3.2.2.2 Antagonist investigations

To determine the effect of TTX, an antagonist of multiple Na_V channels, on DEP induced depolarisation of the vagus nerve, the standard antagonist protocol was followed. Two reproducible control responses to DEP (1ug/ml) were first obtained, and the nerve allowed to fully wash before a ten-minute incubation with TTX (3µM). Immediately following the incubation period, the nerve was challenge with DEP in the presence of TTX and the inhibition was calculated. The nerve would then be washed out with Krebs, before a final application of DEP applied to confirm nerve viability at the end of the experiment.

3.2.2.3 Analysis

At the beginning of the experiment, the chart recorder was calibrated so that 1mm was equal to 0.01mV. Vagal nerve depolarisation was measured in mV as read from the chart recorder graph paper. For antagonist studies, depolarisations that occurred in the presence of antagonist drugs were compared against the average depolarization of the prior control responses in the same tissue to calculate antagonist % inhibition. Statistical calculations of antagonist effects were calculated using a paired two tailed t-test. Data is presented as mean ± S.E.M, with statistical significance set at $P < 0.05$.

3.3 Results

3.3.1 Effect of DEP on firing of single airway sensory nerve fibres *in vivo*

To determine whether DEP can activate airway sensory nerves, initial experiments focused on the effect of intratracheal dosing of DEP in the anesthetized guinea pig.

Table 3.1: Activation profile of DEP on guinea pig airway single fibres *in vivo.*

'Yes' indicates that nerve was activated by the stimuli.

	C-Fibre (CV =< 1m/s)	Aδ-Fibre (CV >= 1m/s)
DEP (10µg/ml)	**Yes (n=3)**	**No (n=4)**
Acrolein (10mM) *TRPA1*	Yes (n=16)	No (n=9)
Capsaicin (100µM) *TRPV1*	Yes (n=54)	Yes/No (n=12/11)

As it was unclear what types of airway sensory nerve fibre were activated by DEP, the nerves investigated were profiled based on sensitivity to chemical and mechanical stimuli. (Table 3.1). In C-fibres, which were not responsive to mechanical stimuli but were activated by capsaicin (100µM) and acrolein (10mM), intratracheal administration of DEP (10µg/ml) induced a clear and sustained pattern of action potential firing, whereas administration of vehicle alone (PBS) did not (Fig 3.1). However, in A-δ fibres, which responded to the mechanical forces of ventilation but not acrolein, DEP (10µg/ml) failed to induce action potential firing. (Fig 3.2).

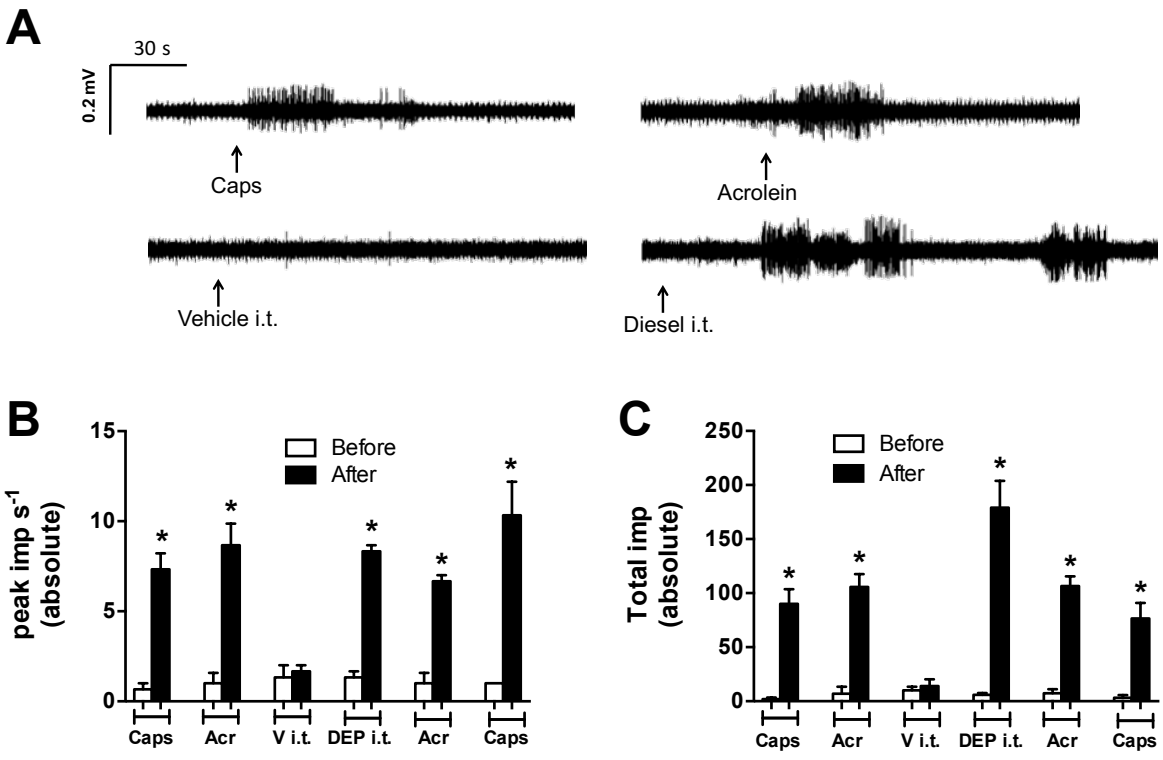


Figure 3.1: Effect of DEP on airway C-fibres *in vivo*.

(A) *Action potential firing in guinea pig airway C-fibre in response to aerosolised capsaicin (100μM in saline, aerosol 15 seconds, top left), acrolein (10mM in saline, aerosol 60 seconds, top right), vehicle (PBS, intratracheal dose, bottom left) and DEP (10μg/ml in PBS, intratracheal dose, bottom right) stimuli. Mean data of the responses to stimuli represented as peak impulses per second* ***(B)*** *and total impulses* ***(C)*** *(n=3). Note that mean data is presented in order as challenged to the animal (first challenge on the left).*

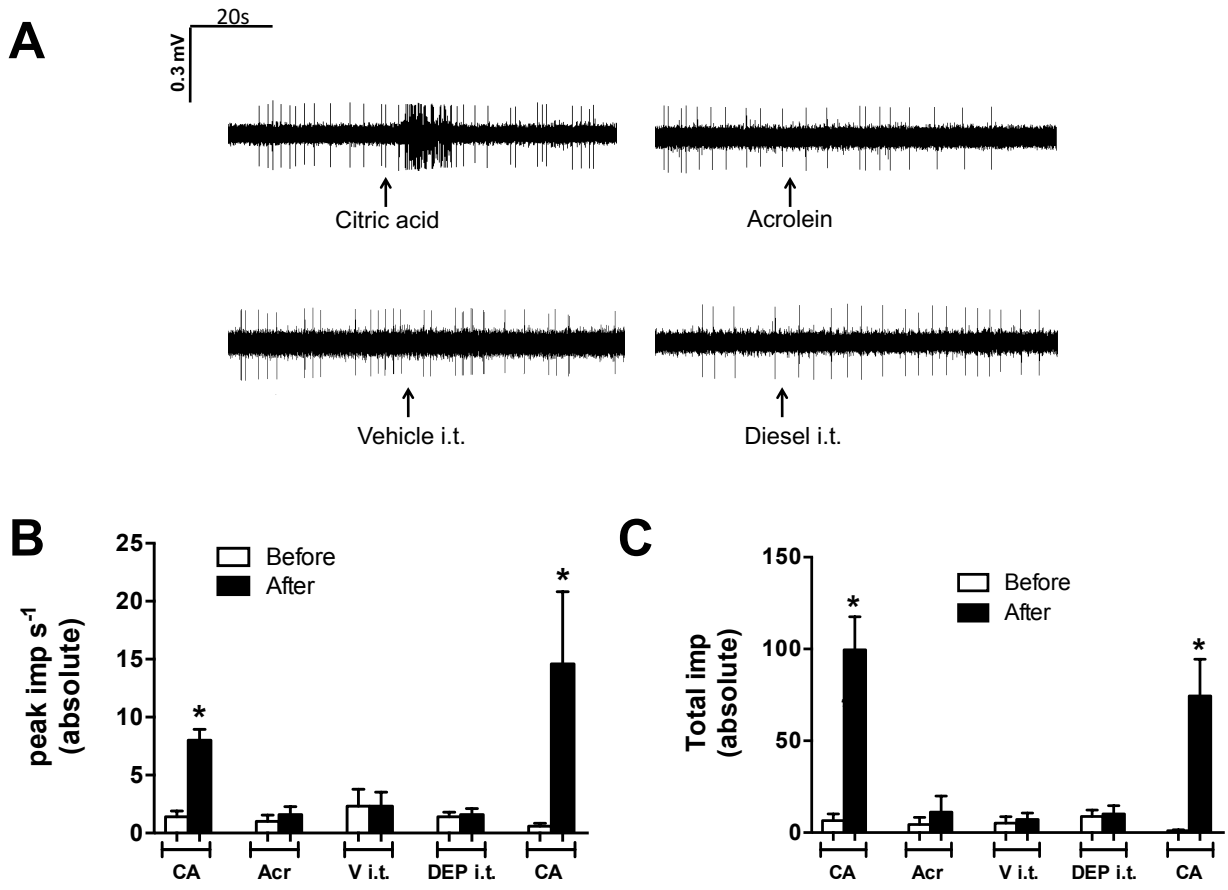


Figure 3.2: Effect of DEP on airway Aδ- fibres sensory nerves in vivo

(A) *Action potential firing in guinea pig airway Aδ- fibre in response to aerosolised CA (300mM in saline, aerosol 60 seconds, top left), acrolein (10mM in saline, aerosol 60 seconds, top right), vehicle (PBS, intratracheal dose, bottom left) and DEP (10µg/ml in PBS, intratracheal dose, bottom right) stimuli. Mean data of the responses to stimuli represented as peak impulses per second* ***(B)*** *and total impulses* ***(C)*** *(n=4, p<0.05, paired t-test). Note that mean data is presented in order as challenged to the animal (first challenge on the left).*

3.3.2 Effect of DEP on depolarisation of isolated vagus nerve

The *in vitro* isolated vagus nerve preparation was used to investigate the effects of DEP on neuronal responses in a higher throughput manner, with the additional benefit that translational data could be obtained from human tissue.

DEP depolarized the guinea pig isolated vagus nerve in a concentration depended manner. DEP (1 µg/ml) induced the maximum recorded depolarization of 0.115 mV ± 0.017 (Fig 3.3B).

This concentration was selected for subsequent antagonist studies as the response to 0.1 µg/ml DEP would have been too small to feasibly carry out pharmacological investigations. DEP (1µg/ml) also induced a response in isolated human vagus nerve (Fig 3.3D).

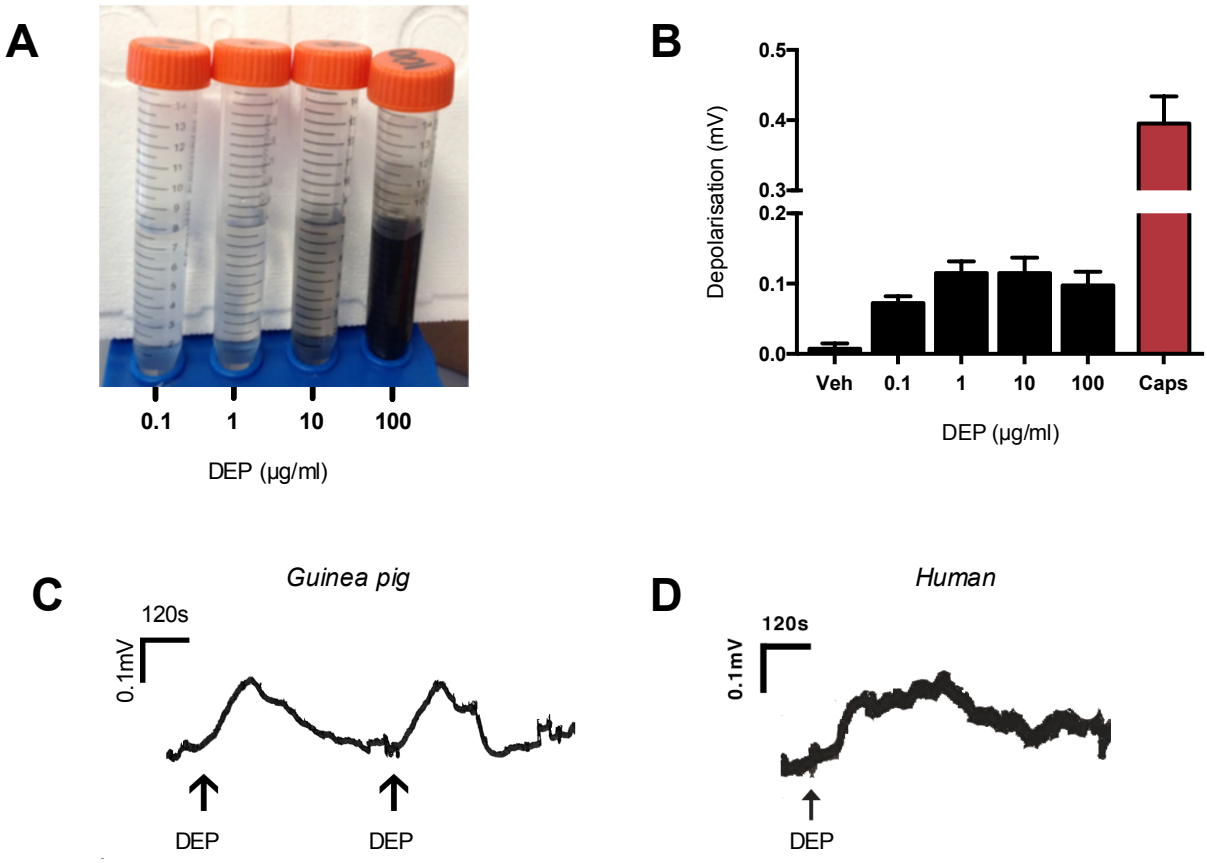


Figure 3.3: Effect of DEP on the isolated vagus nerve

(A) *Photograph showing the concentrations of DEP selected for in vitro studies.* ***(B)*** *Concentration dependent DEP induced depolarisation of the isolated guinea pig vagus nerve, with capsaicin (1µM) shown as a positive control. (n=4)* ***(C)*** *Representative trace showing the depolarization of the guinea pig vagus nerve induced by DEP (1µg/ml).* ***(D)*** *Representative trace showing the depolarization of the human vagus nerve induced by DEP (1µg/ml). Data is expressed as mean ± SEM.*

3.3.3 Effect of TTX on DEP induced depolarisation of the isolated vagus nerve.

To confirm that the observed responses were the results of classical neuronal activation, the effect of the non-selective Na^+ channel inhibitor TTX was investigated. Application of TTX (3µM) completely inhibited the responses to DEP (1µg/ml), and after washout the nerve again responded to DEP challenge (Fig. 3.3)

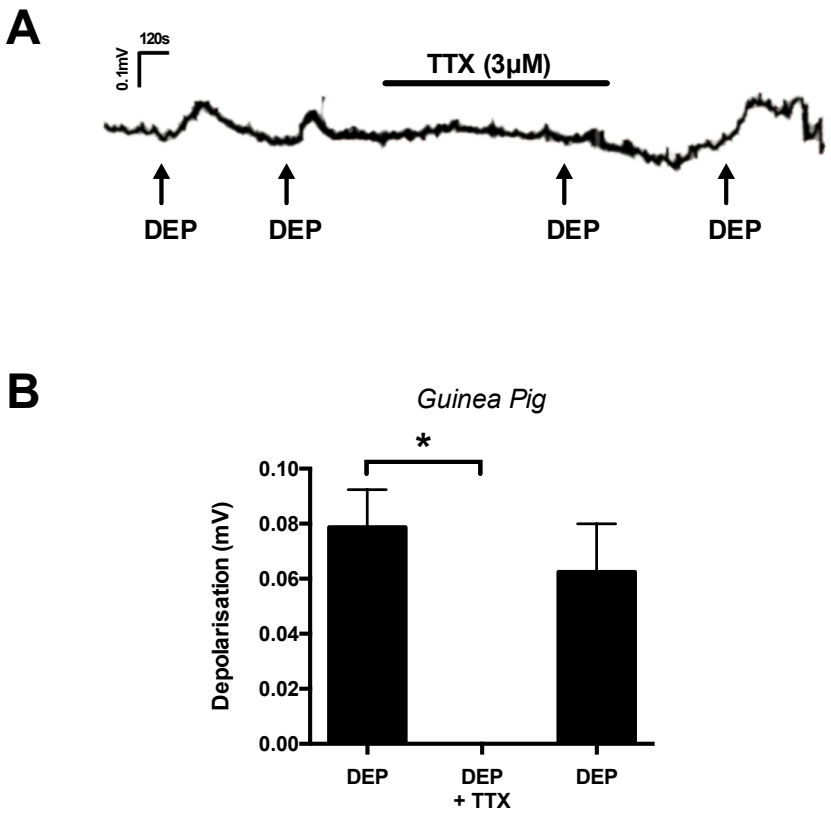


Figure 3.4: Effect of TTX on DEP induced depolarisation of isolated guinea pig vagus nerve

(A) *A representative trace showing the effect of TTX (3μM) on DEP (1μg/ml) induced depolarisation.* ***(B)*** *Mean data shown graphically, with the left column indicating the control responses to the DEP, the middle column indicating the responses to DEP in the presence of TTX and the right hand column indicating the recovery responses to DEP after washout of TTX (n=4, p<0.05 paired t-test). Data is expressed as mean ± SEM.*

3.4 Discussion

The aim of this chapter was to determine whether DEP is capable of activating airway sensory nerves both in *vivo* and *in vitro*. DEP are one of the major components of urban PM (Shah *et al.*, 2004), especially in Europe where diesel engines are found in a significant number of vehicles on the road (Cames & Helmers, 2013) and are generated from the combustion of diesel fuel from engines and generators. It has been well established that there are respiratory health risks associated with exposure to DEP (McClellan, 1987; Kagawa, 2002; Kelly & Fussell, 2011; Ristovski *et al.*, 2012). Black smoke, a proxy measure of DEP, has also been associated with reduced lung function, cough and wheeze in children living closest to

motorways (Brunekreef *et al.*, 1997; Vanvliet *et al.*, 1997). Further epidemiological studies have linked DEP exposure with increased hospital admission for respiratory issues (Samoli *et al.*, 2016). More controlled clinical studies have shown similar findings with increases in airway resistance reported upon exposure to DEP (Stenfors *et al.*, 2004; Carlsten *et al.*, 2014).

However, the precise mechanisms by which DEP drives such adverse respiratory effects are not clear. Although there is a large body of work that has focused on the effects of DEP on components of the respiratory system, the majority of studies have focused on interactions with epithelial or immune cells (Maier *et al.*, 2008). Inflammatory mediators such as Nf-κB have been shown to be released from human bronchial epithelial cells (Takizawa *et al.*, 1999), as well as GM-CSF and IL-8 (Boland *et al.*, 1999). DEP has been shown to induce matrix metalloprotease expression in A549 cells (Amara *et al.*, 2007). DEP can alter gene expression in macrophages (Verheyen *et al.*, 2004), as well as impair their ability to deal with pathogen infection (Yang *et al.*, 2001).

However, one potential mechanism that has not yet been explored is the role of airway sensory nerves in the adverse health effects of DEP. Airway sensory nerves that innervate the airways are capable of detecting noxious stimuli and initiating a variety protective respiratory reflexes such as cough and bronchoconstriction (Canning *et al.*, 2006; Nasra & Belvisi, 2009), which are similar to the reported symptoms that occur upon exposure to DEP (McCreanor *et al.*, 2007). Vagotomy of rats reduced the level of inflammation of the rat lung when challenge with diesel soot (McQueen *et al.*, 2007), hinting that the vagus nerve may indeed play a role.

In this present study, DEP was administered *in vivo* to anaesthetised guinea pigs. It should be noted that although delivering aerosolized DEP would have been the preferred method to match real world exposure scenarios, there were practical limitations in terms of the compatibility of existing aerosolisation hardware and the use of particulate matter. As such, an intratracheal bolus of DEP dispersed in PBS was administered to the animal instead. Action potential firing in single airway afferents were recorded as a measure of neuronal activity in response to DEP challenge. It was shown that DEP only activated C-fibre afferents but not Aδ- fibre afferents. These first key results have shown that DEP is capable of activating airway sensory nerves in a live animal. In a healthy individual, respiratory reflexes are protective responses designed to protect that airways from harm (Canning *et al.*, 2006), however it is now clear that their activation can drive a range of respiratory disease pathologies (Coleridge & Coleridge, 1984a; Belvisi, 2003a; Nasra & Belvisi, 2009). Given this link, further investigation is necessary to understand the contribution of exposure to DEP to such issues.

As an initial starting point in terms of mechanistic investigation, the selective sensitivity of different subtypes of airway afferents to DEP already reveals some potential insights. Although the classification of airway sensory nerves is complex, Aδ-fibres can be broadly characterised as being sensitive to mechanical stimuli (such as physical touch or the stretch that occurs during ventilation of the lung), while C-fibres are thought to be more chemosensitive. It has been shown that some of the biological effects of DEP, such as the induction of CYP1A1, can be attributed to the physical presence of the particle itself (Totlandsdal *et al.*, 2012), however in the same study other cytokines such as IL-6 and IL-8 were upregulated by the presence of the organic chemicals embedded on the surface DEP. Other studies confirm that the organic fraction is capable of exerting inflammatory effects within cells (Li *et al.*, 2002; Hirano *et al.*, 2003; Siegel *et al.*, 2004). In agreement with these findings, airway afferents that were sensitive to chemical stimuli were shown to be activated by DEP, rather than mechanically sensitive afferents. These results indicate that it is likely to be certain organic chemicals on the surface of DEP that are capable of activating airway sensory nerves, rather than the physical presence of carbon particulate matter in the airways.

With the activation of airway sensory nerves by DEP confirmed *in vivo*, the effect of DEP was then studied *in vitro*. The isolated vagus nerve technique is an alternative method of investigating sensory nerve activation that benefits from reducing the time, complexity, number of animals used and cost of experiments performed, making it more amenable to the pharmacological investigations necessary to understand the mechanisms involved in DEP induced activation of sensory nerves (Birrell *et al.*, 2009; Bonvini *et al.*, 2015). Another major benefit of this technique is that human vagal tissue can be obtained from lung tissue that is surplus to transplant requirements, and thus translational data can be obtained from key results obtained from animal models. However, some limitations do exist. Isolated vagus nerve recordings are not made of direct action potential firing, but instead measure the summation of nerve depolarisation in the whole trunk of the vagus nerve. Furthermore, the vagus nerve innovates various parts of the body in addition to the lungs and airways, so responses observed cannot strictly be established as ones that would occur in the airways. However, a large number of studies within the Respiratory Pharmacology group have shown that agonists that depolarize the vagus nerve *in vitro* will also cause action potential firing of airway afferents *in vivo* (Birrell *et al.*, 2009; Maher *et al.*, 2009).

It was shown that DEP was capable of depolarizing the isolated guinea pig vagus nerve in a concentration dependent manner. From this concentration response, 1μg/ml was selected a suitable concentration for subsequent antagonist investigations. It should be noted that this concentration is significantly lower than levels selected for many *in vitro* cell based assays

(Lehmann *et al.*, 2009; Chaudhuri *et al.*, 2012; Totlandsdal *et al.*, 2015), highlighting either the sensitivity of the neuronal response or the inappropriate concentrations elsewhere. Furthermore, simple visual observation of different concentrations of DEP added weight to the idea that experiments utilising concentrations in excess of 100µg/ml may have limited real world exposure relevance, given the opacity of the solutions concerned. It was also confirmed that DEP (1ug/ml) was capable of depolarising human vagus nerve tissue, suggesting that the activation of DEP was preserved across species and thus results obtained from animal tissues would be of use progressing knowledge of the human health impacts of DEP.

To verify that the observed depolarisation was a true neuronal response, the effect of fast voltage gated sodium channel (Na_V) blocker TTX was investigated. In the classical description of an action potential, local changes in the membrane potential of a nerve will cause it to depolarise from a resting membrane potential state (Stuart *et al.*, 1997). If a sufficient level of depolarisation occurs then the threshold potential across the membrane is reached and at this point Na_V channels are activated, causing a rapid influx of Na^+ ions into the localised area of the cell (Catterall, 2000). This causes a further rapid further depolarisation which is sufficient to depolarise the adjacent areas of the cell membrane and cause neighbouring Na_V channels to be activated, thus triggering an action potential as the depolarisation signal propagates along the nerve. TTX, a non-selective Na_V channel inhibitor, can be used as a pharmacological tool to confirm that that Na_V channels are involved in any observed responses (Cestèle, 2000). In this study, it was shown that TTX (3µM) completely abolished the responses to DEP. Together with the *in vivo* single fibre recordings, these *in vitro* results confirm that DEP is capable of activating the vagus nerve.

Before further investigations into the biological effects of DEP on airway sensory nerves can be carried out, it is first necessary to understand its characteristics. Unlike a conventional drug or endogenous substance, particulate matter such as DEP can have an extremely varied composition (Vogt *et al.*, 2003). Numerous factors such as fuel type and operating conditions can alter the physicochemical properties of DEP, and individual particles will differ in morphology and composition (Ålander *et al.*, 2004). SRM-2975 was selected as the DEP of choice as it originates from a single source and thus is more standardised than a real world sample, but nevertheless it is important to characterise the DEP that are being used in this present study. Additionally, it will be possible to identify the presence of organic chemicals that potentially play a role the observed responses. Therefore, in the next chapter, I will investigate the physicochemical properties of DEP and look to identify which components are responsible for the observed activation of airway sensory nerves.

4 Chapter Four: Physicochemical characterisation and determining the critical components of DEP

4.1 Rationale

In the previous chapter, it was shown that DEP can activate airway sensory nerves. DEP activated the chemically sensitive C-fibres, but not the mechanically sensitive A-δ fibre afferents, suggesting that chemical compounds present in DEP may be responsible for the observed activation.

Previous studies have demonstrated that a variety of properties of DEP can have an effect on biological systems (BeruBe *et al.*, 1999). DEP can form agglomerates several micrometres in aerodynamic size, but single primary particles can be as small as 20nm in diameter and be classified as airborne nanoparticles (Park *et al.*, 2004; Tetley, 2007). Smaller particles, especially those measured in the nanometre range, can drive biological responses to PM or other elements that are traditionally non-toxic at the micrometre scale (Alvaro R Osornio-Vargas, 2003; Chen *et al.*, 2006). In terms of composition, DEP contains a carbon core surrounded by organic hydrocarbons and embedded trace metals (Matti Maricq, 2007). The presence of transition metals can cause inflammation and DNA damage within the lung (Lingard *et al.*, 2005; Molinelli *et al.*, 2008; Lim *et al.*, 2009). The organic components of DEP contain a range of chemical compounds, including PAHs that have adverse biological effects (Kim *et al.*, 2013), and may be responsible for the activation of C-fibres. These organic extractions containing hydrocarbons present in DEP have also been shown to induce cytokine release in bronchial epithelial cells and inward Ca^{2+} flux in dorsal root ganglia (DRG) neurons (Li *et al.*, 2002; Baulig *et al.*, 2003a; Totlandsdal *et al.*, 2015).

The precise composition of DEP is highly complex and can vary based on a multitude of factors including engine load and fuel type (Ålander *et al.*, 2004; Ratcliff *et al.*, 2010). Given that the physicochemical composition of DEP mediates any potential biological effects, it is clear that appropriate characterisation must be carried out for meaningful interpretations of

biological data to be made (Warheit, 2008). In this present chapter the physicochemical composition of DEP will be investigated; including particle size, metallic content and inorganic/organic carbon composition. Additionally, the effect of the organic compounds present within DEP will be investigated with regards to their role in the observed activation of vagal afferents.

4.1.1 Chapter Hypothesis:

DEP is formed from nanometre sized primary particles and larger agglomerates. Embedded hydrocarbons on the surface of DEP are responsible for neuronal depolarisation.

4.1.2 Aims:

- Measure the particle size of DEP using dynamic light scattering (DLS) and cryogenic-electron microscopy (cryo-EM).
- Analyse the metallic content of DEP using energy dispersive X-ray spectroscopy in the transmission electron microscope (TEM-EDX).
- Quantify the relative amount of organic hydrocarbons present in DEP using thermogravimetric analysis (TGA).
- Extract the organic hydrocarbons in DEP using Soxhlet extraction.
- Compare the effects of the organic components of DEP to the carbon core using the isolated vagus nerve preparation.

4.2 Methods

4.2.1 Particle size measurements

4.2.1.1 DLS measurements of DEP

DLS is an analytical technique that calculates the size distribution of particles in a sample by measuring fluctuations of light intensity caused by the Brownian motion of particles in a liquid (Bihari *et al.*, 2008; Murdock *et al.*, 2008). DEP samples were analysed by DLS using a Zetasizer Nanoseries Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Approximately 0.8ml of DEP (0.1µg/ml and 10µg/ml in Krebs) was placed into semi-micro disposable plastic Poly(methyl methacrylate) cuvettes (VWR International - UK). The

Zetasizer was heated to 37°C, then the cuvette was placed inside the machine and allowed to thermally stabilise for 2 minutes before particle size measurements are taken using a scattering angle of 173°. Each measurement consisted of 10 fifteen-second repeats, with triplicate measurements being taken on 3 separate days using freshly made solutions.

4.2.1.2 Cryo-EM imaging of DEP

DEP (1µg/ml in Krebs) was dropped onto a grid and frozen by rapidly plunging into liquid ethane. The imaging grid was then transferred in a frozen state into a cryo-rod (Gatan 914) and then into a TEM (Jeol 2100F) operated at 200kV. Due to the irregular shape and size of the particles, size analysis was carried out by measuring the longest length dimension of particles and agglomerates for 394 particles.

4.2.2 Metallic species characterisation

4.2.2.1 TEM Energy Dispersive X-Ray (TEM-EDX)

To determine the elemental profile of DEP, TEM-EDX analysis was carried out. TEM-EDX utilises the unique spectra of X-Rays emitted by specific elements after bombardment with an electron beam, to identify the elemental composition of a sample (Niemi *et al.*, 2006). DEP was dispersed by sonicating for 60 seconds in ethanol and aliquoted onto copper grids before imaged using an FEI Titan 80–300 TEM operated at 300 kV. EDX was carried out by condensing the electron beam onto the region of interest and acquiring spectra for 180 seconds.

4.2.3 Organic component quantification and testing.

4.2.3.1 TGA of DEP

The organic/inorganic ratio composition of DEP was assessed using TGA. TGA is a commonly used thermal analysis technique that measures changes in mass during controlled heating of a sample and has previously been utilised to study the composition of DEP (Stratakis, 2003; Lapuerta *et al.*, 2007). TGA was carried out using a Perkin Elmer Pyris 1 machine (PerkinElmer Inc., Beaconsfield, UK) by heating 1.8 ± 0.2 mg of DEP to 100 °C under air flow (flow rate 10 mL/min), and holding isothermally for 30 minutes to remove residual water and/or

solvent; the temperature was then increased from 110 °C to 850 °C at a constant ramping rate of 10 °C/min under air flow (10 mL/min).

4.2.3.2 Separation of inorganic/organic components of DEP

To determine whether activation of sensory nerves by DEP was driven either by the presence of the inorganic carbon core or organic hydrocarbons, the two components in the DEP were separated using soxhlet extraction. Soxhlet extraction is a well established technique used to separate soluble fractions from insoluble fractions in a sample (Luque de Castro & Priego-Capote, 2010).

1g of DEP was loaded into a paper filter and extracted overnight with dichloromethane (DCM) using soxhlet extraction equipment at 40°C. The extract was then evaporated by rotary distillation, and reconstituted in DMSO up to a volume of 10ml. This resulted in an 'equivalent concentration' of 100µg/ml, whereby the organic content contained within 1g of DEP was now present in a known volume of solvent. The reconstituted DEP organic extract (org-DEP) was centrifuged at 10,000g for 20 minutes to remove any remaining particulates and the supernatant was carefully removed. Org-DEP was then diluted in DMSO to a working concentration of 1µg/ml and stored at -80°C until needed. The remaining particles left behind in the Soxhlet paper filter were heated gently overnight to completely evaporate any remaining DCM, and were then removed and kept as cleaned particles (par-DEP). Par-DEP was prepared for *in vitro* experiments in a similar manner to DEP, as described in Chapter 3, whereby a stock solution of par-DEP (500µg/ml in Krebs) was sonicated for 10 minutes before diluting to a working condition of 1µg/ml in Krebs.

4.2.3.3 Determining responses to separated components of DEP

Vagus nerve isolated from male Dunkin Hartley guinea pigs and from tissue from lung donors that was surplus to transplant requirements were placed into a grease gap recording chamber to record the vagal nerve depolarisation, described in Chapter 2.

To compare the effects of the separated components of DEP, the isolated guinea pig vagus and human vagus nerves were stimulated with org-DEP, par-DEP and DEP (each 1 µg/ml in Krebs) for 2 minutes and the resulting vagal depolarisation recorded. After stimulation, the vagus nerve was washed with Krebs solution until the recordings returned to baseline level and the next stimulation was applied. In addition to investigating the effects of materials

generated within our laboratory, the effects of commercially available materials were also investigated. A concentration response was carried out by applying non-cumulative concentrations of either vehicle (0.1% DMSO) or a commercially organic extract of DEP (SRM-1975, NIST, USA; 0.1µg/ml to 100 µg/ml as calculated as an 'equivalent concentration' in a similar manner to org-DEP) in a randomised manner to the isolated vagus nerve. Following each two minute stimulation, the nerve was washed with Krebs solution until response returned to baseline, after which the next concentration was tested. A concentration response to either vehicle (Krebs) or carbon black (CB; 0.1µg/ml to 10µg/ml), an analogue for the carbon core of DEP, was also carried out in a similar manner.

4.3 Results

4.3.1 Particle size measurements

4.3.1.1 DLS measurements of DEP.

The average hydrodynamic particle size as measured by % intensity of the DLS signal was calculated as 251.5nm ± 16.1 for DEP (0.1µg/ml) and 508.2 ± 20.8 for DEP (1µg/ml) (Fig 4.1A). Analysis of the frequency distribution indicated that all particles were below 1µm in size (Fig 4.1B). Comparing the two concentrations of DEP analysed, demonstrated that an increase in concentration was accompanied by an increase in the average hydrodynamic diameter of the particles. Over the time period taken to complete three triplicate measurements, the hydrodynamic diameter also increased indicating the rapid agglomeration of particles in solution (Fig 4.1C).

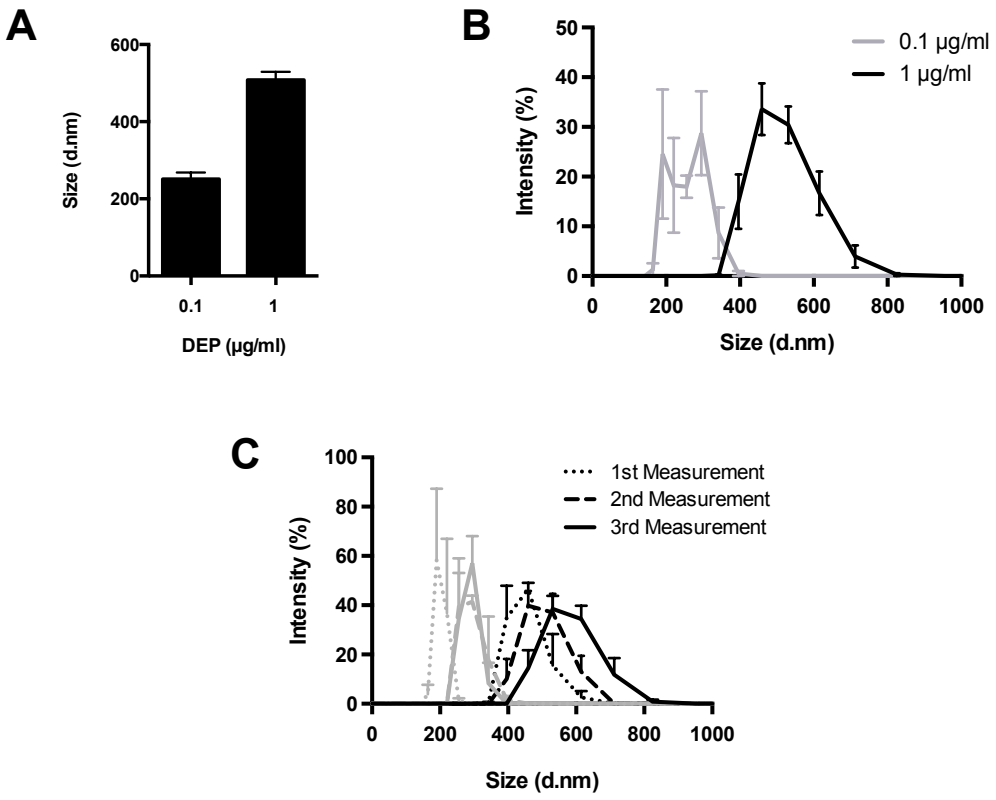


Figure 4.1: Hydrodynamic diameter size of DEP measured by DLS.

DEP was suspended in Krebs and sonicated prior to DLS measurements. ***(A)*** *The mean diameter was measured as 251.5nm ± 16.1 for DEP (0.1µg/ml) and 508.2 ± 20.8 for DEP (1µg/ml)* ***(B)*** *The frequency distribution particle size distribution was measured by % intensity of DLS signal at two concentrations of DEP (0.1µg/ml and 1µg/ml).* ***(C)*** *Effect of time on particle size distribution. Data presented as mean ± SEM. Measurements were carried out on three separate days (n=3).*

4.3.1.2 cryo-EM imaging of DEP

As a technique more suitable for particles suspended in aqueous solution, cryo-EM was utilised to image particles for assessment of morphology and manual measurement of primary particle size. Cryo-EM revealed small aggregates of various sizes (Fig 4.2A). The mean particle size was measured as 298.7nm ± 13.9, with the median size measured as 232.1nm, although some larger aggregates were measured at >1µm in size (Fig 4.2B).

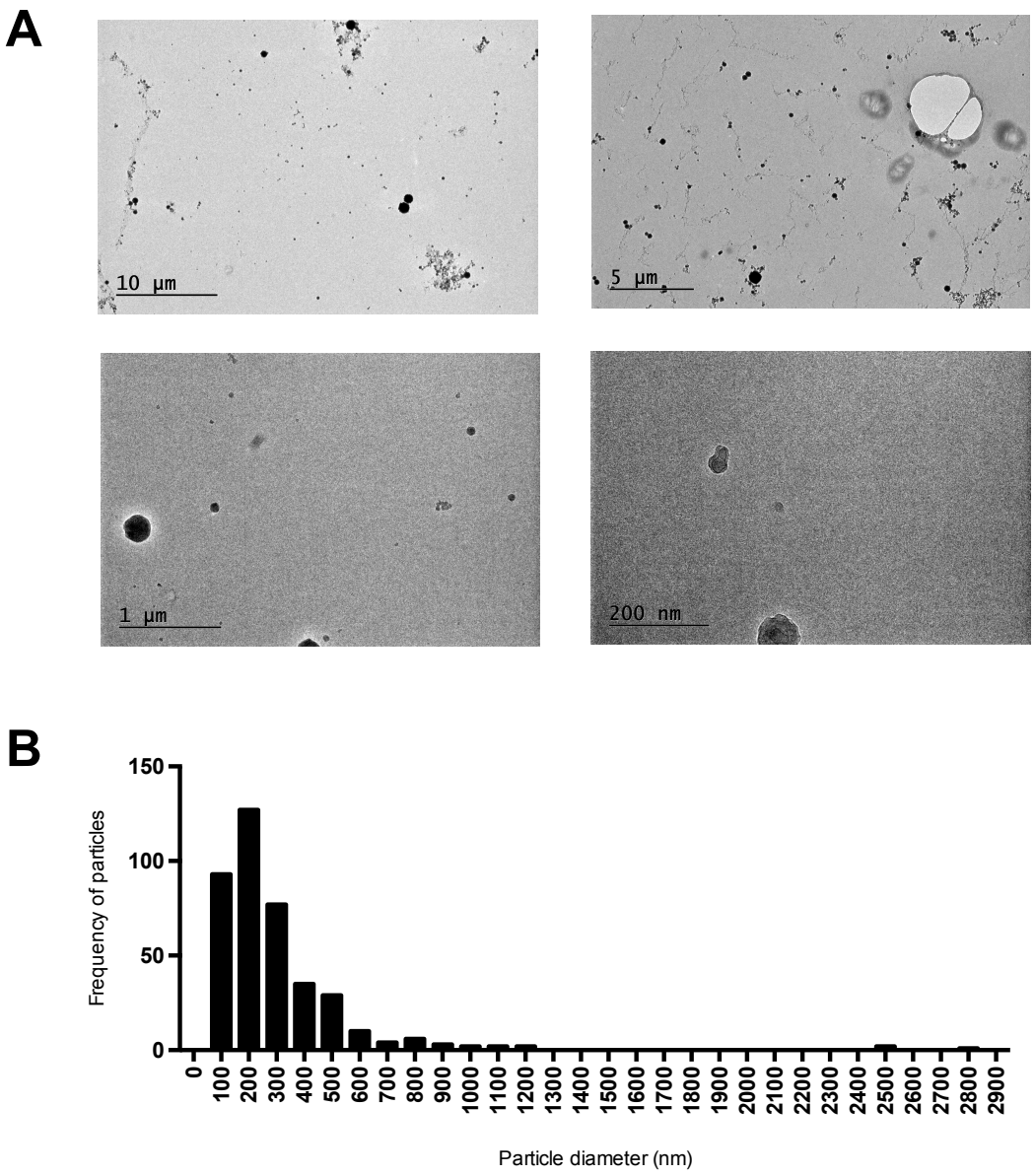


Figure 4.2: Cryo-EM imaging of DEP.

DEP (1µg/ml) were dispersed in Krebs, and imaged using cryo-EM. ***(A)*** *Example images of DEP at various scales of magnification.* ***(B)*** *Size frequency distribution of particles and agglomerates of DEP as measured by longest dimension (n=394).*

4.3.2 Metallic species characterisation

4.3.2.1 TEM-Energy Dispersive X-ray (TEM-EDX)

TEM-EDX revealed an absence of significant metallic species in the sample of DEP analysed. In the blank control, elemental carbon (C), oxygen (O), copper (Cu) and silicon (Si) were detected (Fig 4.3A). During the analysis of the DEP sample the same elements were detected,

with the addition of trace levels sulphur (Cu) and calcium (Ca) (Fig 4.3C). The presence of Cu in both spectra was the result of the copper grid that samples were aliquoted onto.

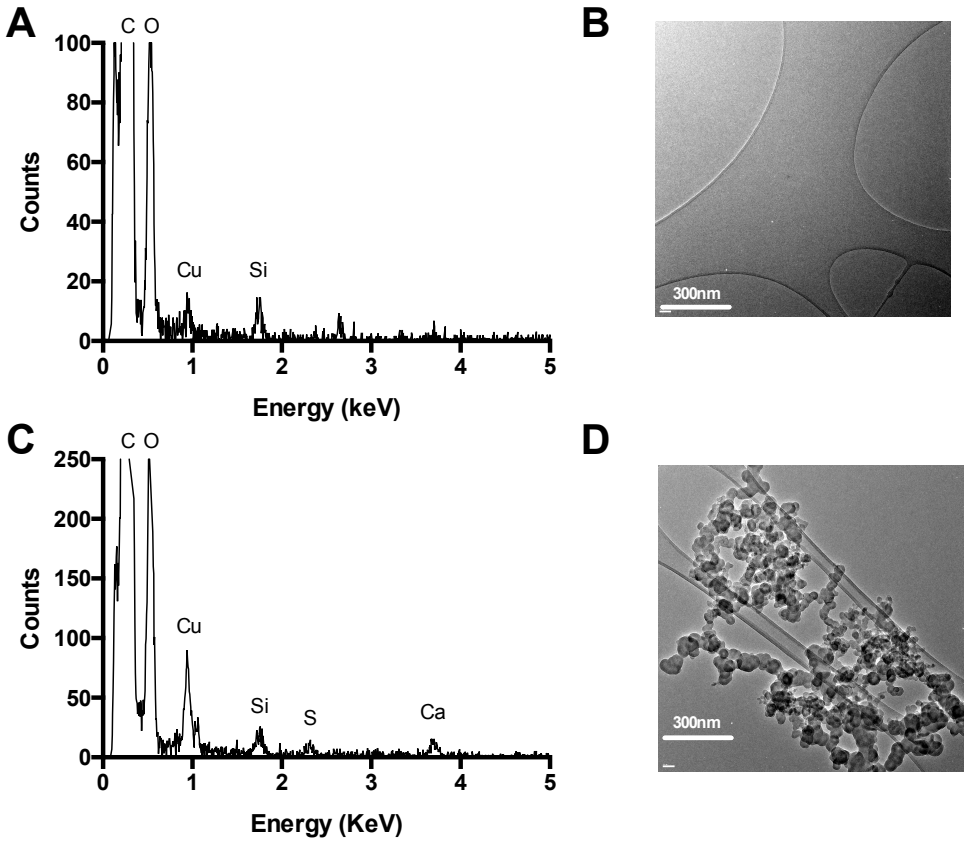


Figure 4.3: TEM-EDX spectrum of metallic species present in DEP.

*DEP were dispersed in ethanol and analysed on a copper grid. **(A)** TEM-EDX spectrum showing elemental carbon (C), oxygen (O), copper (Cu) and silicon (Si) as detected in the negative blank control. **(B)** Location imaged for negative blank analysis. **(C)** TEM-EDX spectrum showing elemental carbon (C), oxygen (O), copper (Cu), silicon (Si), sulphur (S) and calcium (Ca) as detected in the DEP sample. **(D)** Location imaged for DEP sample.*

4.3.3 Organic component quantification.

4.3.3.1 TGA weight loss profile of DEP

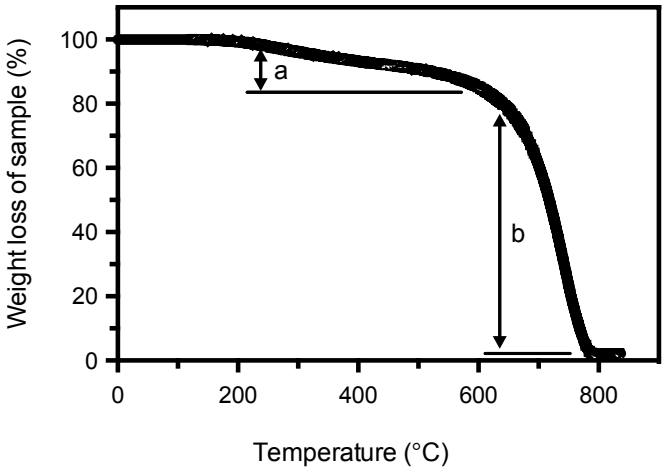


Figure 4.4: Thermogravimetric profile of DEP.

TGA weight loss profile of DEP when heated to 850 °C in air, showing (a) organic component fraction and (b) inorganic carbon fraction lost during heating.

TGA revealed that 15% of the mass of the DEP sample was lost between 200°C and 600°C, corresponding to the degradation temperatures of the more volatile organic chemical component (Figure 4.4). As the sample was continued to be heated to 800°C, 83% of mass was lost representing the inorganic carbon component. The remaining 2% that remained was attributed to trace impurities.

4.3.4 Comparison of the effects of the components of DEP on depolarization of the isolated vagus nerve.

With minimal metallic content, the two significant components of DEP were the organic components and the carbon core. Soxhlet extraction of DEP was carried out to separate the organic components of DEP (org-DEP) from the cleaned particulate carbon core (par-DEP). Only org-DEP (1 µg/ml), but not par-DEP (1 µg/ml), activated the guinea pig vagus nerve (Fig 4.5A). This activation was of a similar magnitude to whole DEP (1µg/ml) (Fig 4.5B). These experiments were paralleled in isolated human vagus tissue and similar results obtained (Fig 4.5C).

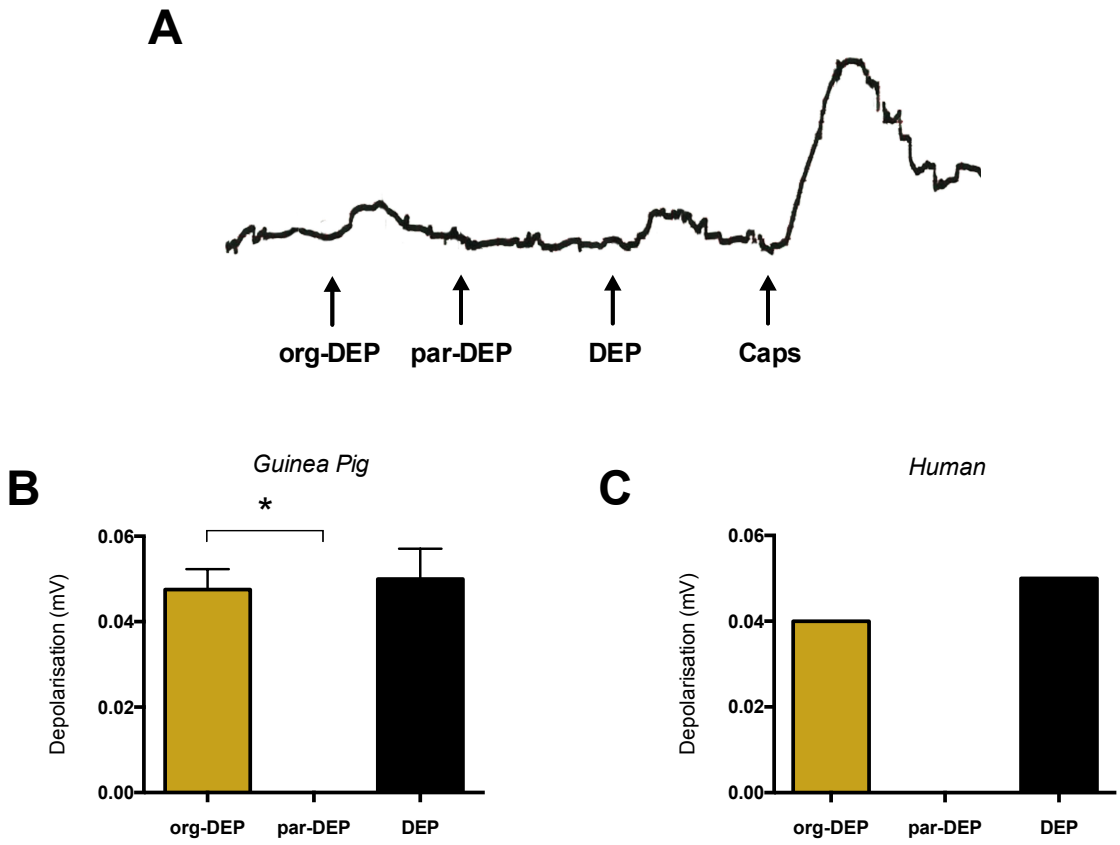


Figure 4.5: Comparing the responses of the components of DEP.

(A) *Representative trace showing depolarisation induced by org-DEP (1µg/ml), par-DEP (1µg/ml) and DEP (1µg/ml) in isolated guinea pig vagal tissue. Caps (1µM) is shown as a positive control. (N=4).* ***(B)*** *Graphical data from guinea pig vagal tissue (N=4) and* ***(C)*** *human vagal tissue (N=2). Data is expressed as mean ± SEM. * denotes statistical significance (P < 0.05), calculated using a paired t-test.*

To confirm the results from 'self-made' extracts, commercially available materials were tested. DEP-OE (SRM 1975, NIST, USA), a commercial available organic extract of SRM-2975, depolarized the isolated guinea pig vagus nerve in a concentration dependent manner, with 1µg/ml producing a depolarisation of 0.13mV ± 0.03 (Fig 4.6A). However, carbon black, an analogue for the carbon core of DEP, failed to depolarize the isolated guinea pig vagus nerve (Fig 4.6B). These findings mirror the results obtained with the 'self-made' separated components of DEP.

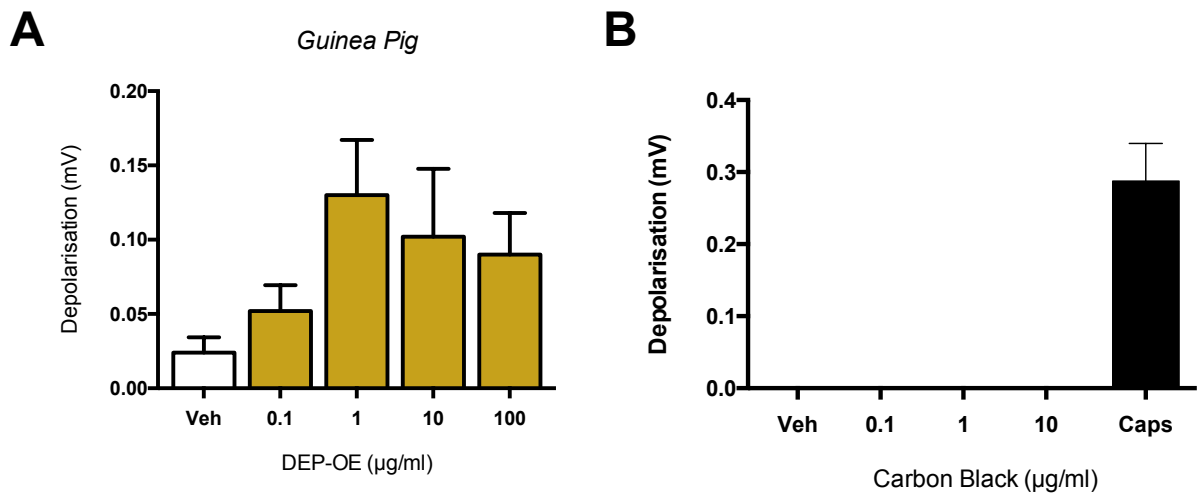


Figure 4.6: Effects of DEP-OE on depolarization

(A) *DEP-OE caused a concentration dependent depolarisation of the isolated guinea pig vagus nerve (N=4-5)* ***(B)*** *Carbon black failed to induce depolarisation of the isolated guinea pig vagus nerve (n=4). Data is expressed as mean ± SEM.*

4.4 Discussion

In the previous chapter it was determined that DEP was capable of activing the vagus nerve. The aim of this current chapter was to characterise the properties of DEP and to identify which components were responsible for the observed activation of the nerve.

Given that DEP were to be utilised in physiological salt solutions (Krebs) for subsequent *in vitro* and *in vivo* studies, initial particle size measurements were carried out using DLS. DLS is a technique that is commonly used to measure the average hydrodynamic diameter of particles when suspended in a liquid media (Murdock *et al.*, 2008). An increase in the average recorded particle size occurred both as a result of an increase in DEP concentration and the short time periods between measurements, indicating that aggregation of DEP was occurring. DEP is known to readily aggregate, with primary nanoparticles subunits forming larger chained structures (Shi *et al.*, 2000; Singh *et al.*, 2004). DEP samples are heterogeneous in particle size, therefore DLS is not an appropriate technique to utilise to study the primary size of the particles, as larger particles dominate the recorded signal and thus skew data erroneously (Khlebtsov & Khlebtsov, 2011). Despite this, DEP were in the expected sub-1µm size range. With the majority of particle sizes measured on the nanometre scale, DEP possess the ability to be inhaled into the lungs and airways (Tetley, 2007; Geiser & Kreyling, 2010). Some of the cardiovascular effects of diesel exhaust exposure have even been proposed to be due to

nanoparticle sized DEP being small enough to cross the alveolar barrier and thus be translocated systemically *via* the bloodstream (Solomon *et al.*, 2013).

As an alternative to DLS, a more appropriate measurement of particle size was carried out using electron microscopy. Traditional TEM techniques would be unsuitable for imaging DEP dispersed in Krebs solution due to the presence of drying artefacts that would occur due to the presence of salts in the physiological media. Cryo-EM, a technique that freezes samples within solutions before EM imaging (Bai *et al.*, 2015), was utilized to image DEP. Rapid freezing of samples allows for preservation of natural morphology and prevents the aforementioned dehydration issues with traditional TEM from occurring (Kuntsche *et al.*, 2011; Milne *et al.*, 2013). Following the acquisition of suitable images, particle size was quantified by measuring the longest length of particles or aggregates. The majority DEP aggregates were found to be below 1 μm, in agreement with results from DLS measurements as well as with previous studies (Singh *et al.*, 2004; Kocbach *et al.*, 2005). When comparing average particle size frequency distributions, DEP (1μg/ml), as measured by cryo-EM was smaller than the same concentration measured by DLS. This discrepancy between the techniques is likely due to the fact that DLS measurements bias towards larger particle sizes in heterogeneous samples. In terms of morphology, DEP aggregates formed chained structures with individual subunits as expected.

One potential mechanism by which DEP exert adverse respiratory effects is through the presence of transition metals found embedded on their surface (BeruBe *et al.*, 1999; Matti Maricq, 2007). Transition metals possess the potential to generate oxidative stress (Charrier & Anastasio, 2012) and thus when carried into the lungs by DEP can initiate a range of cellular responses, with cytotoxicity and increased inflammatory mediator release observed in human bronchial epithelial cells exposed to DEP with greater metallic content (Totlandsdal *et al.*, 2015). Removal of metals from PM attenuates the release of IL-8 from human epithelial cells (Molinelli *et al.*, 2008). Tracheal installation of DEP deposits iron into the lungs of rats, depleting lavage antioxidant ascorbate concentrations, elevating macrophage levels and increasing inflammatory mediators (Ghio *et al.*, 2000). Neuronal cells are sensitive to metallic nanoparticle uptake, with cell death occurring in DRG neurons upon exposure to copper nanoparticles and subsequent mitochondrial dysfunction (Scott, 2012). Zinc, cadmium and copper have also been shown to directly induce Ca^{2+} influx and activate airway sensory neurons (Gu & Lin, 2010). From the results of TEM-EDX analysis in this chapter, this present sample of DEP was found to be have minimal metallic content. These results are in agreement in studies elsewhere demonstrating that SRM-2975 is a relatively ‘clean’ DEP in terms of transition metal content, with other studies showing that only 0.22% of the weight of SRM-

2975 was attributable to metal content (Ball *et al.*, 2000; Jensen, 2006). It seems unlikely therefore that the activation of airway sensory nerves by DEP observed in this thesis is driven by the presence of transition metals.

In the previous chapter, DEP selectively activated chemically sensitive C-fibres *in vivo.* Studies have highlighted the importance of the organic chemical components of DEP in driving cellular responses (Li *et al.*, 2002; Baulig *et al.*, 2003a; Totlandsdal *et al.*, 2015). For this reason, it was important to characterise the ratio of organic to inorganic carbon in DEP using TGA. TGA is a commonly used thermal analysis technique, whereby changes on mass can be detected upon heating a sample in a controlled atmosphere (Stratakis, 2003; Lapuerta *et al.*, 2007). DEP was found to be approximately 15% organic material, with around 83% inorganic carbon and, in keeping with the results TEM-EDX analysis, few trace impurities. These results are in rough agreement with Singh *et al.,* (2004) whereby the carbon/organic ration of SRM-2975 was found to 60:5 by % mass.

Soxhlet extraction was carried out to determine the role of the main components of DEP by separating the organic compounds (org-DEP) and inorganic carbon core (par-DEP) present in DEP. The effects of these components on the isolated vagus nerve were determined. To validate the findings from these 'self-made' extractions the effects of a commercially available organic extracts of DEP (DEP-OE) and carbon black (CB), acting as an analogue for the carbon core, were also investigated. Existing studies in the literature are conflicting, with some results indicating that the carbon core of DEP is capable of exerting biological effects. CB is cytotoxic to epithelial cells (Murphy *et al.*, 1999) and upregulates the expression of heme oxygenase (HO-1), a sensitive marker for oxidative stress, in alveolar macrophages (Koike & Kobayashi, 2006). However, other investigations have concluded inorganic carbon is biologically inert and that the organic components of DEP drive biological responses. In human bronchial epithelial cells, DEP and organic extracts of DEP were capable of inducing the release of pro-inflammatory cytokines while CB had no effect (Boland *et al.*, 1999; Baulig *et al.*, 2003b). In this present study, the organic extracts of DEP, not the inorganic carbon core or CB, was capable of depolarising the vagus nerve at similar concentrations to that of DEP in Chapter 3. Together these results show that the observed activation of airway sensory nerves by DEP is driven by the organic components of DEP.

In summary this chapter has characterised DEP the physicochemical properties of DEP. Confirming the initial hypothesis, the organic component was found to drive the observed activation of sensory nerves. The next chapter will focus on identifying whether TRP channels, as environmental sensors, are mediating DEP induced activation of airway afferents.

5 Chapter Five: Identifying the role of TRP channels in DEP induced activation of airway sensory nerves

5.1 Rationale

In the previous chapter, DEP was physicochemically characterised and it was demonstrated that the organic components of DEP (DEP-OE) were responsible for the observed depolarisation of the vagus nerve. Having identified which are the active components of DEP, further investigation is necessary to elucidate the cellular mechanisms underlying the activation of the vagus nerve. Vagal depolarisation requires the activation of an ion channel, allowing the influx of ions into the cell to alter the membrane potential. However, the exact channels responsible for such depolarisation in response to DEP is not known. In the vagus nerve, transient receptor potential (TRP) channels have been shown to act as environmental sensors that can be activated by a wide range of endogenous and exogenous stimuli (Pedersen *et al.*, 2005). While the evidence is limited, a few studies have shown that can be activated by DEP. TRPV1 has been implicated in DEP induced oedema in the rat airways (Teles *et al.*, 2009). DEP has been shown to cause Ca^{2+} influx via TRPV4 in human bronchial epithelial cells (Li et al., 2011). DEP also similarly activated TRPA1 in murine dorsal root ganglion cells (Deering-Rice *et al.*, 2015). It is important to note that activation of these TRP channels has been shown to activate airway sensory nerves to initiate reflex responses (Adcock, 2009; Birrell *et al.*, 2009; Bonvini *et al.*, 2015; 2016), and interestingly many of these reflex events lead to the respiratory symptoms associated with DEP exposure (Vanvliet *et al.*, 1997; Nordenhäll *et al.*, 2000; Sydbom *et al.*, 2001). Therefore, it is possible that activation of airway sensory nerves by DEP occurs via the activation of TRP channels and this chapter will investigate this hypothesis.

5.1.1 Chapter Hypothesis:

The activation of airway sensory nerves by DEP is mediated by the activation of TRP channels.

5.1.2 Aims:

- To confirm that TRPA1, TRPV1, and TRPV4 are expressed on airway sensory nerves. This will be investigated through the use of RT-PCR techniques in both the whole ganglia and individually isolated airway innervating neurons.
- To determine the correct concentration of TRP antagonists (Xention D0501 - TRPV1; GSK2193874 – TRPV4; Janssen 130 – TRPA1) to utilise for pharmacological investigation by carrying out a concentration response of the antagonists against their specific agonist in the isolated vagus nerve preparation.
- Investigate the effects of TRP antagonists on DEP induced depolarisation of the isolated vagus nerve utilising both guinea pig and human tissue.
- Investigate the role of TRP channels on DEP induced depolarisation using isolated vagus nerve tissue obtained from TRP channel knock-out mice.
- Investigate the effects of TRP channel antagonists against DEP induced action potential firing *in vivo* using single airway afferent firing recordings in the guinea pig.

5.2 Methods

5.2.1 mRNA expression of TRP channels

5.2.1.1 Whole ganglia quantitative RT-PCR

Quantitative RT-PCR primers were purchased for TRPA1 (Cp04230138_m1), TRPV1 (Cp03755294_m1) and TRPV4 (Cp4230394_m1) (Applied Biosciences). These primers had been previously validated using cDNA generated from tissues in the guinea pig that expressed significant levels of the target channel of interest. Whole nodose and jugular ganglia were harvested from male Dunkin Hartley guinea pigs and real time RT-PCR was performed, as previously described in Chapter 2, to measure expression of TRPA1, TRPV1 and TRPV4.

5.2.1.2 Single Cell RT-PCR

Male Dunkin Hartley guinea pigs were intranasally dosed with a retrograde tracer dye DiI. 14 days later, the nodose and jugular ganglia were enzymatically digested and neurons cultured overnight on flurodishes coated with laminin. Airway innervating neurons were identified as those having taken up the dye using a Widefield light microscope, and were individually harvested from the culture dish using a custom made pipette and placed into separate

autoclaved Eppendorf tubes, in which. Quantitative RT-PCR reactions could then be carried out to determine the expression of TRP channels (Primers listed in Table 5.1). PCR products were separated by means of electrophoresis on 1.5% agarose gels. Results were deemed valid by the absence of products in the negative water controls and also when products were obtained from the PCR reactions using β-actin (forward 5' – 3': TGGCTACAGTTTCACCACCA; reverse 3' – 5': GGAAGGAGGGCTGGAAGA) and PGP 9.5 (forward 5' – 3': GCCAGTGTCGGGTAGATGAC; reverse 3' – 5': CGTGTGTGCAGAACCAAAGG) primers as positive controls, indicating successful picking of a neuronal cell.

Table 5.1: Quantitative RT-PCR TRP primers

	Forward	Reverse
TRPA1	5'- CATTTTGCTGCAACCCAAGGAGCCACTG -3'	5'- ATAAGTGGAGAGCGTCCTTCAGAATC -3'
TRPV1	5'- CCAACAAGAAGGGGTTCACA -3'	5'- ACAGGTCATAGAGCGAGGAG -3'
TRPV4	5' – TGGGCAAGAACTCAGATGGC– 3'	5' - TCCACAGTCCTAGAGGGGAG– 3'

5.2.2 Genotyping of genetically modified mice

Genetically modified mice were bred on a background of C57BL/6 mice purchased from Harlan, (United Kingdom). TRPV1$^{-/-}$ breeding pairs were backcrossed on to the C57BL/6 background and were obtained originally from Jackson Laboratories (Bar Harbour, Maine, USA). TRPA1$^{-/-}$ mice were originally developed by Professor David Julius (University of California, San Francisco, USA) and supplied as backcrossed 10 generations on to C57BL/6 background by Professor Peter Zygmunt from Lund University. Homozygous breeding pairs of mice genetically modified to disrupt the TRPV4 gene were obtained from Riken BioResource Center (Tsukuba, Japan). Mice were bred and housed at Imperial College London (Central Biomedical Services).

To confirm the expected deletion or modification of the TRP channels of interest, genotyping of the mice was carried out as previously described in Chapter 2. Tail tips from male C57Bl/6 wild-type (WT) controls and TRP$^{-/-}$ mice were removed and DNA samples were extracted. Regions of interest were amplified using TRP primer sequences purchased from Invitrogen (Table 5.2) and Promega GoTaq Flexi PCR reagents.

Table 5.2: PCR primers for genotyping

	Forward	Reverse	Neo
TRPA1	5'-AGCTGCATGTGTGAATTAAATTCTTAGCA-3'	5'-ATCACCTACCAGTAAGTTCATGAGAACA-3'	N/A
TRPV1	5'-CCTGCTCAACATGCTCATTG-3'	5'-CCTGCTCAACATGCTCATTG-3'	5'-CACGAGACTAGTGAGACGTG-3'
TRPV4	5' – TGT TCG GGG TGG TTT GGC CAG GAT AT– 3'	5' - GGT GAA CCA AAG GAC ACT TGC ATA G– 3	5' – TGG ATT GCA CGC AGG TTC TC – 3'.

The PCR protocol began with 1 cycle of 95°C for 120 secs, before the individual protocols detailed in Table 5.3. The protocol finished with a final cycle of 72°C for 10 mins and then 4°C for 5 mins. PCR products were visualised on a 2% agarose gel (80V for 1 hour) in TBE buffer (0.05 µl/ml Safeview – NBS Biological Ltd, Huntingdon, UK) alongside ladder controls of the appropriate size for the expected products (Hyperladder, Bioline Reagents, UK).

Table 5.3: PCR Protocol for *TRP*$^{-/-}$ mice

	PCR Protocol				Product Size (bp)	
	Cycles	**Denaturing**	**Annealing**	**Extension**	**WT**	**KO**
TRPA1	40	95^{o}C *30 sec*	52^{o}C *30 sec*	72^{o}C *60sec*	296	142
TRPV1	40	95^{o}C *30 sec*	64^{o}C *60 sec*	72^{o}C *60sec*	984	600
TRPV4	40	95^{o}C *30 sec*	60^{o}C *30 sec*	72^{o}C *80sec*	740	1490

5.2.3 Isolated vagal nerve recordings

Vagus tissue isolated from male Dunkin Hartley guinea pigs, WT C57Bl/6 mice and TRP$^{-/-}$ KO mice and where possible human donor tissue was placed into a grease gap recording chamber to record vagal nerve depolarisation, as previously described in Chapter 2.

5.2.3.1 Antagonist concentration response

While there are various TRP channel antagonists available, for this thesis one compound was selected for each TRP channel. Janssen 130 (Jan 130) was selected as a TRPA1 antagonist (Berthelot *et al.*, 2013), Xention D0501 (Xention) was selected as a TRPV1 antagonist (Round *et al.*, 2011) and GSK2193874 (GSK219) was selected as a TRPV4 antagonist (Thorneloe *et al.*, 2012). To identify the correct concentration of these selective TRP antagonists to be used against DEP-OE induced responses, firstly a concentration response was carried out against their respective agonists (see Table 5.4) following the standard antagonist experimental protocol. Agonists were applied to the vagus nerve for 2 minutes to produce a control response before washing with Krebs until reaching baseline. This agonist stimulation was repeated a second time to get a reproducible response. Next, the nerve was treated with the antagonist of interest for ten minutes. Then the nerve was again challenged with the agonist in the presence of the antagonist and the resulting depolarisation recorded. This antagonist treated response was then compared to the average of the control responses. Finally, the system was washed out and the nerve challenged with the agonist a final time to generate a recovery response to confirm nerve viability. If nerves did not respond at the end of the experiment, then the result was discarded. Vehicle (0.1% DMSO) was also tested to confirm it did not have an effect on TRP agonist responses.

Table 5.4: Concentrations of TRP agonists and antagonists.

	Agonist	Concentration	Antagonist	Concentration range
TRPA1	Acrolein	300μM	Jan 130	0.01, 0.1, 1, 10 (μM)
TRPV1	Capsaicin	1μM	Xention	0.1, 1, 10, 100 (nM)
TRPV4	GSK101	300nM	GSK219	0.3, 1, 3, 10 (μM)

5.2.3.2 Effect of TRP antagonists on DEP-OE and DEP induced responses

To determine the effect of these specific TRP antagonists on DEP-OE (1μg/ml) induced vagal depolarisation, the standard antagonist experimental protocol was followed. The vagus nerve was challenged with two repeated stimulations of DEP-OE to obtain baseline recordings, then the nerve treated with the TRP antagonist of interest for 10 minutes. Jan 130 (10μM) was used

to investigate the role of the TRPA1 channel. Xention (100nM) was used to investigate the role of the TRPV1 channel. GSK219 (10μM) was used to investigate the role of the TRPV4 channel. Following antagonist treatment, the nerve was challenged again with DEP-OE in the presence of the antagonist. After a suitable washout period with Krebs, the nerve was re-challenged with an end of experiment stimulation of DEP-OE to confirm tissue viability. If nerves did not respond at the end of the experiment then the result was discarded. Vehicle (0.1% DMSO) was also tested to confirm it did not have an effect on DEP-OE induced responses. As well as investigating the effect of antagonists on DEP-OE induced responses, the above protocol was also carried out against DEP (1μg/ml).

5.2.3.3 Effect of DEP-OE in genetically modified mouse vagal tissue

To complement the data generated from pharmacological studies, vagal tissue from WT (C57Bl/6) and TRP$^{-/-}$ mice was challenged with 2 minute stimulations of DEP-OE (1μg/ml) and TRP agonists in a randomized manner. Following stimulation, the nerve was washed again with Krebs to baseline, then challenged with the next stimulation. For vagal tissue from TRPA1$^{-/-}$ mice, acrolein (300μM) was used as a positive control, while capsaicin (1μM) was used as a negative control. For TRPV1$^{-/-}$ mice, capsaicin (1μM) was the positive control while acrolein (300μM) was the negative control. For TRPV4$^{-/-}$ mice, GSK101 (300nM) was the positive control while capsaicin (1μM) was the negative control.

5.2.3.4 Analysis

At the beginning of the experiment, the chart recorder was calibrated so that 1mm was equal to 0.01mV. Vagal nerve depolarisation was measured in mV as read from the chart recorder graph paper. For antagonist studies, depolarisations that occurred in the presence of antagonist drugs were compared against the average depolarisation of the prior control responses in the same tissue to calculate antagonist % inhibition. Statistical calculations of antagonist effects were calculated using control responses and antagonist responses in the same tissue, therefore a paired two tailed t-test was utilised. For experiments utilising TRP$^{-/-}$ vagal tissue, depolarisations in WT controls were compared with those from TRP$^{-/-}$ tissue. As comparisons were made across tissue from different animals statistical calculations were carried using an unpaired t-test. Data is presented as mean ± S.E.M, with statistical significance set at $P < 0.05$.

5.2.4 Airway single fibre recordings

From the results obtained with the *in vitro* experiments, key experiments were repeated *in vivo*. Guinea pig single fibre recordings were carried out as previously described in Chapter 3. Guinea pigs were anaesthetised and paralysed, and following surgery the vagus nerve was cut on both sides before the left vagus nerve was pared down to a single nerve fibre from which action potential firing could be measured. From the results obtained in Chapter 3, it was shown that DEP selectively activated C-Fibres. Therefore, investigations into the effect of TRP antagonists on DEP-OE induced firing was only carried out in C-Fibres, as determined by a number of classification criteria including activity in response capsaicin challenge and a lack of activity in response to the mechanical stretch of the lung that occurs during tidal breathing.

2.1.1.1 Effect of TRPA1 antagonist on DEP-OE firing in vivo

After the identification of a suitable C-fibre, control responses were first obtained to obtained to capsaicin (100μM in saline, aerosolized for 15s), acrolein (10mM in saline, aerosolized for 60s) and DEP-OE (1 μg/ml in saline, aerosolized for 60s) prior to the intraperitoneal administration of Jan 130 (30mg/kg, 1% methyl cellulose in saline) 60 minutes before challenging again with capsaicin, acrolein and DEP-OE.

2.1.1.2 Analysis

A paired t-test was used to compare the number of peak impulses per second recorded immediately following the capsaicin, acrolein and DEP-OE challenges in the absence of the Jan 130 against those in the presence of Jan 130. The data is expressed as mean +/- S.E.M, with statistical significance set at $P < 0.05$.

5.3 Results

5.3.1 TRP channel mRNA gene expression

5.3.1.1 Whole ganglia TRP channel mRNA expression

The neurons that innervate the airways have their cell bodies housed within the nodose and jugular ganglia. TRPA1, TRPV1 and TRPV4 were all found to be expressed at the mRNA level

in both the jugular and nodose guinea pig whole ganglia, as determined by quantitative RT-PCR (Figure 5.1).

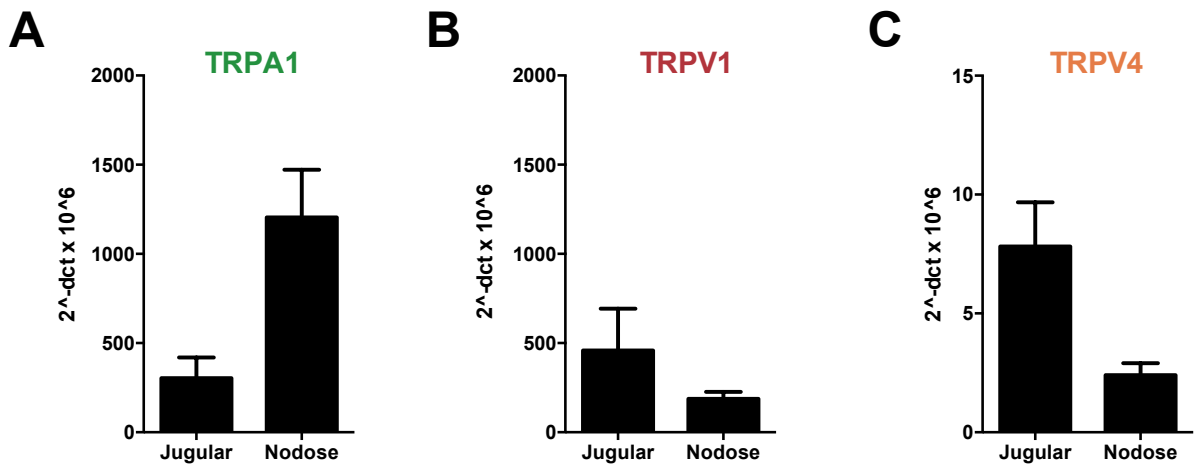


Figure 5.1: TRP channel mRNA expression in whole jugular and nodose ganglia. *Quantitative RT-PCR revealed that TRPA1, TRPV1 and TRPV4 mRNA were all expressed in both the whole jugular and nodose ganglia (n=6-8). Data is expressed at mean ± SEM and normalised to the 18s control.*

5.3.1.2 Single Cell RT-PCR

The whole ganglion contains other cell types in addition to the airway innervating neurons. Therefore, as a more refined technique, single cell quantitative RT-PCR was carried out using individual airway neurons located within the jugular and nodose ganglia. As can be seen in Table 5.5, jugular neurons expressed combinations of TRPA1 and TRPV1 either alone or together, but did not express TRPV4. Nodose neurons also expressed combinations of TRPA1, TRPV1 and a single cell that expressed TRPV4.

Table 5.5: TRP channel gene expression in single airway innervating jugular and nodose neurons.

	TRPA1 alone	**TRPV1 alone**	**TRPV4 alone**	**TRPA1 +TRPV1**	**TRPA1 +TRPV4**	**TRPV1 +TRPV4**	**TRPA1 + TRPV1 + TRPV4**
Jugular neurons	5	5	0	6	0	0	0
Nodose neurons	11	6	0	7	0	0	1

5.3.2 *In vitro* isolated vagal nerve recordings

5.3.2.1 Determining appropriate concentrations of selective TRP antagonists

Having confirmed that the TRP channels of interest were expressed on the mRNA level within airway sensory nerves, the next objective was to use pharmacological tools to assess the potential role that these channels play in mediating DEP-OE induced activation of the vagus nerve. These investigations were firstly carried out using the *in vitro* isolated vagus nerve technique as it has a relatively higher throughput compared to *in vivo* techniques. Initial experiments focused on selecting appropriate concentrations of selective TRP antagonists. A concentration response of Jan 130 (TRPA1 antagonist), Xention (TRPV1 antagonist) and GS219 (TRPV4 antagonist) was carried out against their respective TRP agonists. In a concentration dependent manner, Jan 130 inhibited depolarisation induced by acrolein (30μM - (Birrell *et al.*, 2009)), Xention inhibited depolarisation induced by capsaicin (1μM - (Grace *et al.*, 2012)) and GSK219 inhibited depolarisation induced by GSK101 (300nM - (Bonvini *et al.*, 2016)) (Figure 5.2). From these results, appropriate concentrations of the TRP antagonists could be selected for subsequent experiments (Jan 130 - 10μM; Xention - 100nM; GSK219 - 10μM)

5.3.2.2 Effect of TRP antagonists on DEP-OE induced depolarisation

A submaximal concentration of DEP-OE (1μg/ml) was selected for antagonist studies. In the isolated guinea pig vagus nerve, treatment with the selective TRPA1 antagonist Jan 130 (1μg/ml) significantly inhibited responses to DEP-OE, reducing depolarisation by 84.3 ± 10.1% (Figure 5.3). The selective TRPV1 antagonist Xention (100nM) and the selective TRPV4 antagonist GSK219 (10μM) had no effect on the vagal depolarisation induced by DEP-OE. Similarly, treatment with vehicle (0.1% DMSO) also did not have any effect. In human vagal tissue, Janssen 130 had a similar effect, inhibiting responses to DEP-OE (1μg/ml) by 81.0 ± 9.6%.

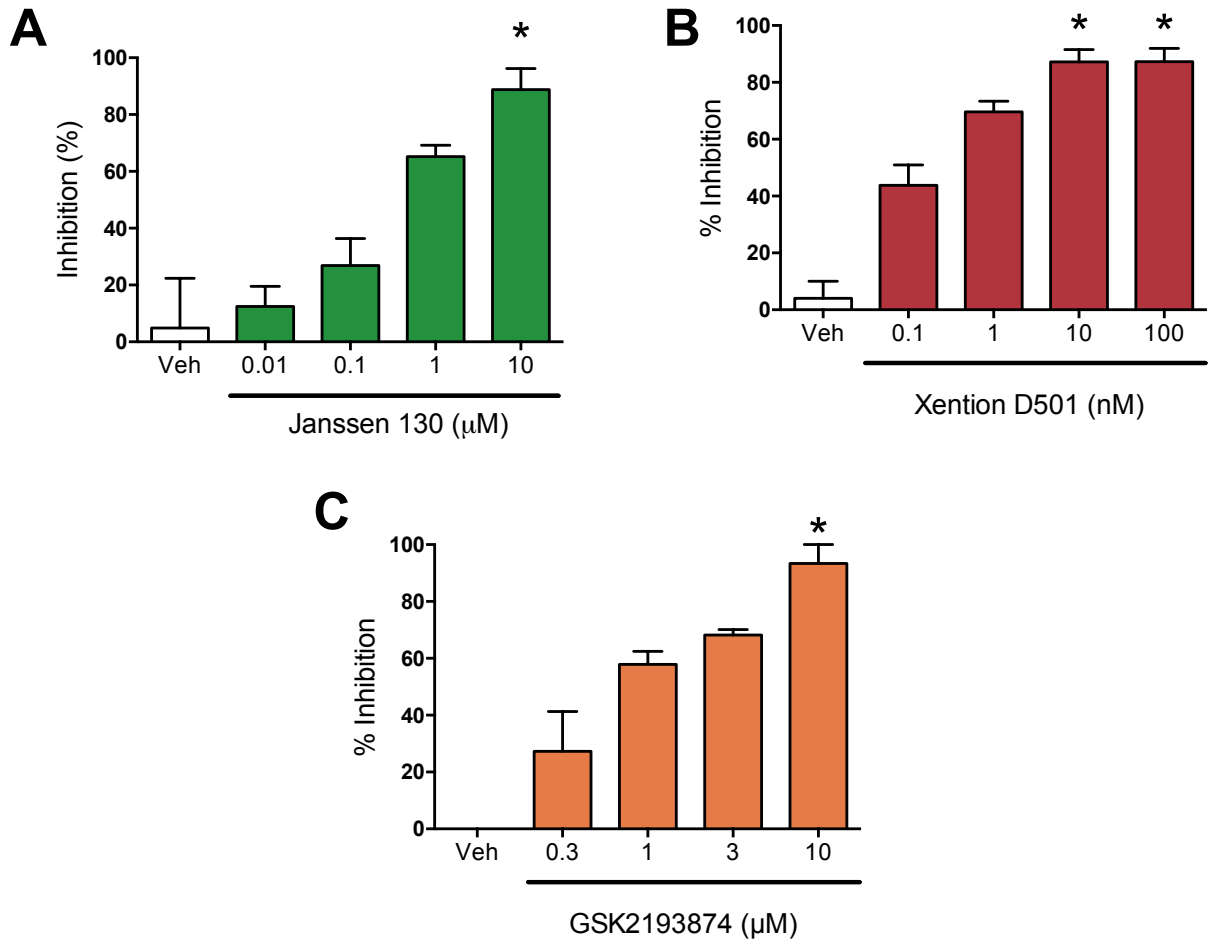


Figure 5.2: Effect of selective TRP antagonists.

*Selective TRP channel antagonists inhibited the depolarisation of their respective agonists in a concentration dependent manner. (A) The TRPA1 antagonist Jan 130 inhibited the depolarisation induced by the TRPA1 agonist acrolein (300μM) (n=4). (B) The TRPV1 antagonist Xention inhibited the depolarisation induced by the TRPV1 antagonist capsaicin (1μM) (n=4). (C). The TRPV4 antagonist GSK219 inhibited the depolarisation induced by the TRPV4 antagonist GSK101 (300nM) (n=4). Data is expressed at mean ± SEM of the percentage inhibition as calculated by comparing agonist responses with in the presence and absence of antagonist within the same nerve. * denotes statistical significance (P < 0.05), calculated using Kruskal Wallis test and Dunns post-test comparing responses to vehicle.*

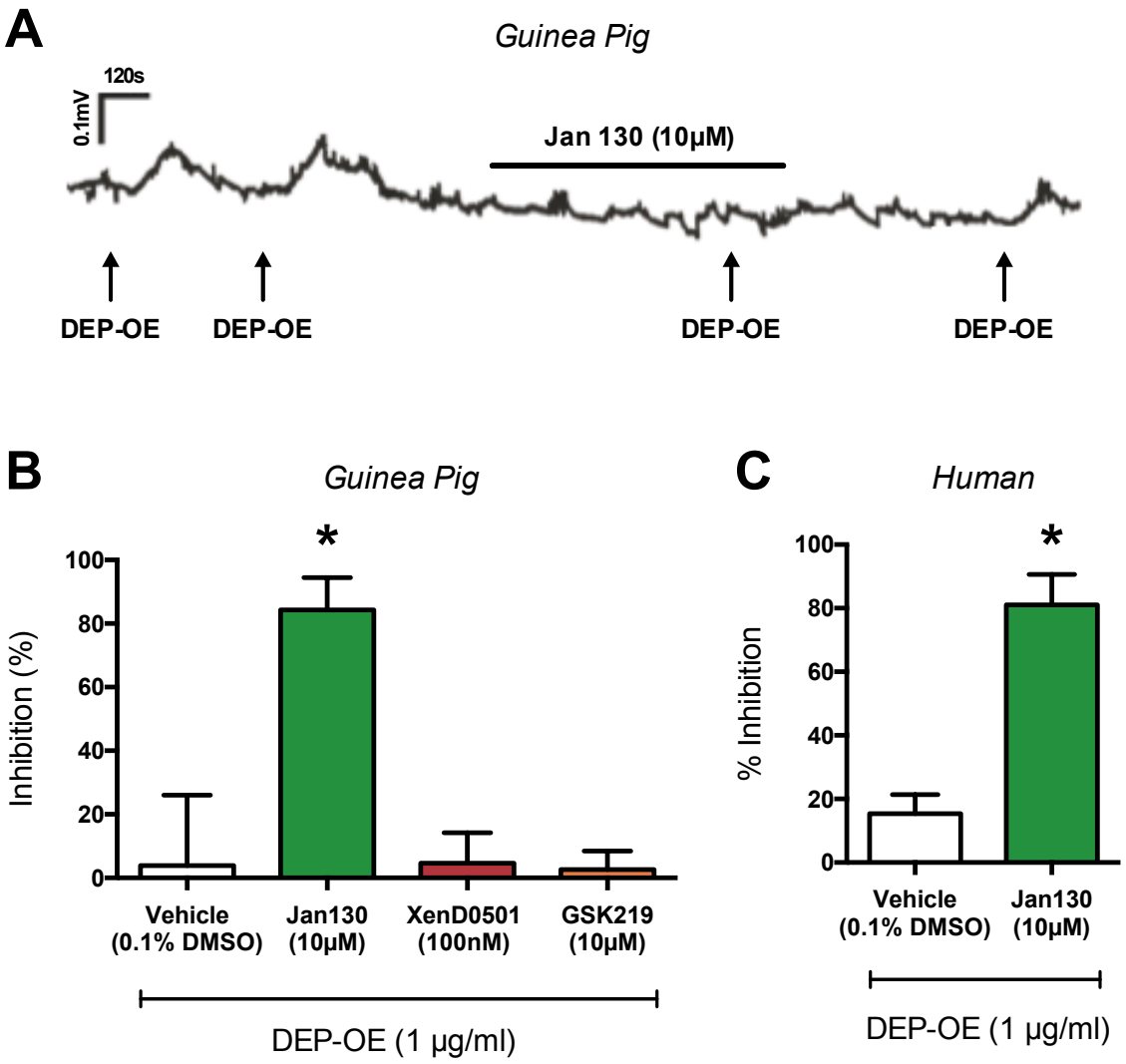


Figure 5.3: Effect of selective TRP antagonists on DEP-OE induced depolarisation.

*Selective TRP channel antagonists were used to investigate the role of TRP channels in the depolarisation induced by DEP-OE. The TRPA1 channel antagonist Jan 130 (10μM) inhibited the response to DEP-OE, with a representative trace shown (A). (B) Histograms showing inhibition of DEP-OE induced responses by Jan 130 (10μM) but no effect from the TRPV1 antagonist Xention (100nM), the TRPV4 antagonist GSK219 (10μM) or vehicle (0.1% DMSO) (n=4-7). (C) Jan 130 also inhibited the depolarisation induced by DEP-OE in human tissue (n=3). Data is expressed as mean ± SEM of the percentage inhibition as calculated by comparing DEP-OE responses with in the presence and absence of antagonist within the same nerve. * denotes statistical significance (P < 0.05), calculated using a paired t-test.*

5.3.2.3 Genotyping of TRP$^{-/-}$ mice

To complement the data generated by pharmacological tools, mice were bred with either genetic modification of either TRPA1 (TRPA1$^{-/-}$), TRPV1 (TRPV1$^{-/-}$) or TRPV4 (TRPV4$^{-/-}$). Before carrying out functional characterisation of the vagal tissues from the mice, firstly the genotype of the mice was confirmed using RT-PCR. Visualisation of the PCR products from these reactions confirmed that mice that were designated as TRP$^{-/-}$ had the expected modifications to their TRP channel sequences (Figure 5.5).

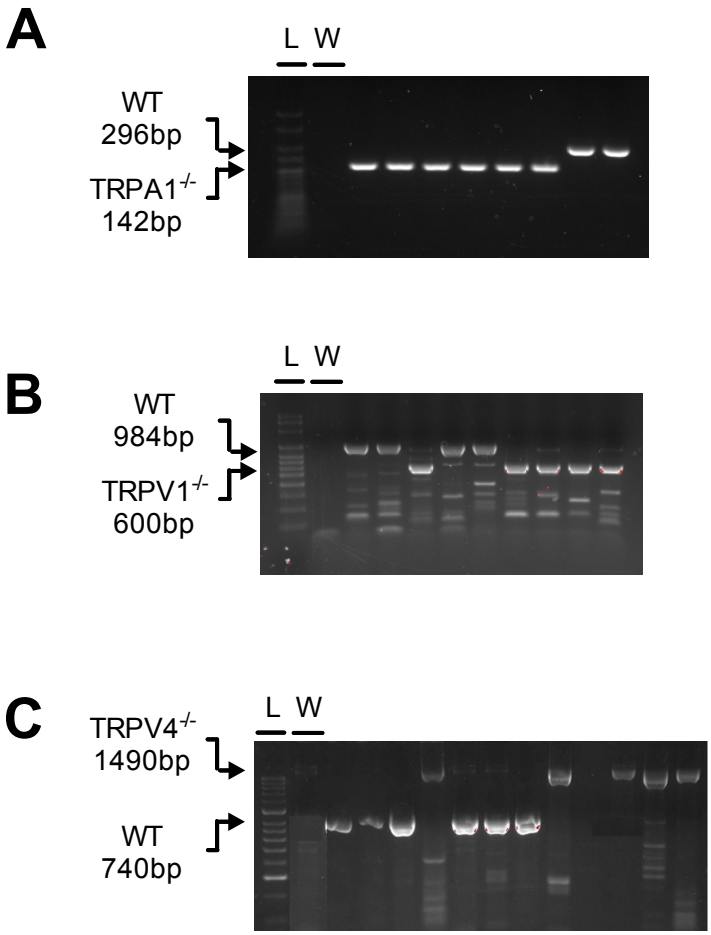


Figure 5.4: Genotyping of TRP KO mice.

RT-PCR genotyping was used to confirm the genetic knockout status of mice using DNA extracted from tail tips. (A) For TRPA1, WT mice formed a product band at 296bp, while KOs at 142bp. (B) For TRPV1, WT mice formed a product band at 984bp, while KOs at 600bp (C) For TRPV4, wild type mice formed a product band at 740bp, while KOs at 1490bp.

5.3.2.4 Functional characterisation of TRP$^{-/-}$ mice

To determine whether the genotype of the TRP$^{-/-}$ mice translated into an expected phenotype, functional characterisation was carried out in the isolated vagus nerve. When compared to WT controls, TRPA1$^{-/-}$ mice showed a significantly reduced response to acrolein (300μM) with a similar lack of response in TRPV1$^{-/-}$ mice to capsaicin (1μM) and TRPV4 mice to GSK101 (300nM) (Figure 5.6). The TRP$^{-/-}$ nerves were also challenged to other TRP agonists as positive controls, confirming nerve viability. These results validated the mouse vagus as a tool to probe the role of TRP channels in DEP-OE induced responses.

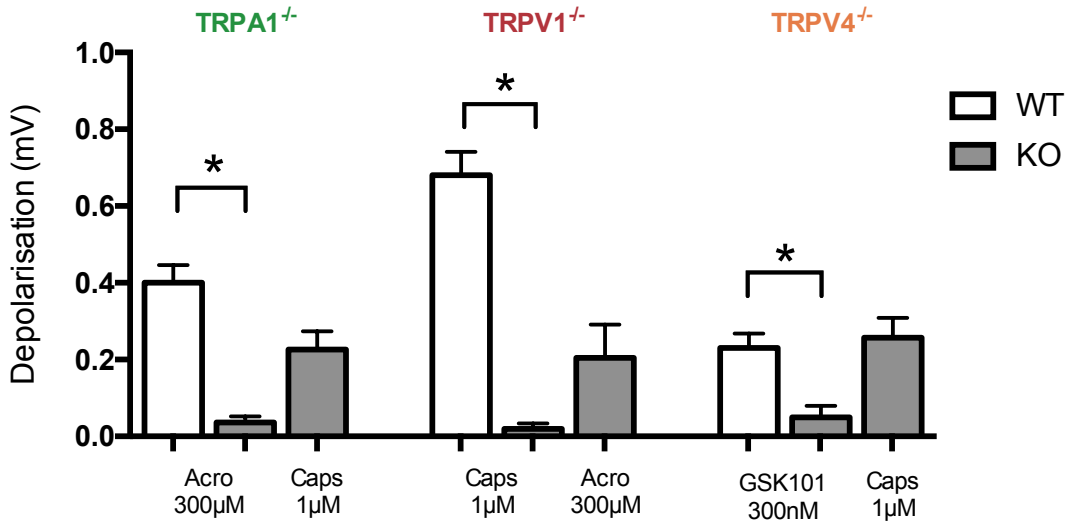


Figure 5.5: Effect of TRP agonists in TRP$^{-/-}$ mice.

*Depolarisation induced by TRP agonists in the vagal tissue of TRP$^{-/-}$ mice (n=4-8). TRPA1$^{-/-}$ mice had reduced responses to acrolein (300uM), TRPV1$^{-/-}$ mice had reduced responses to capsaicin (1uM) and TRPV4$^{-/-}$ mice had reduced responses to GSK101 (300nM). Positive controls also shown to demonstrate nerve viability. Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05), calculated using an unpaired t-test.*

5.3.2.5 Effect of DEP-OE in TRP$^{-/-}$ mice

Vagal tissue from TRP$^{-/-}$ mice were challenged with DEP-OE (1μg/ml). Depolarisation induced by DEP-OE were abolished in TRPA1$^{-/-}$ mice, but were unaltered by TRPV1$^{-/-}$ or TRPV4$^{-/-}$ mice as compared to WT controls (Figure 5.7). These results mirrored the pharmacological results, indicating that TRPA1 is the key ion channel mediating responses to DEP-OE.

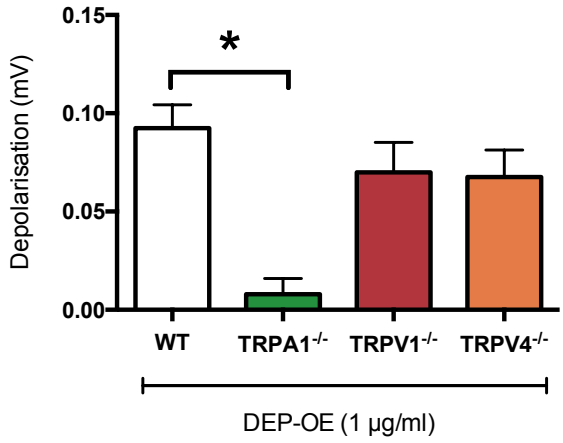


Figure 5.6: Effect of DEP-OE in TRP KO mice.

*Depolarisation induced by DEP-OE in the vagal tissue of TRP$^{-/-}$ mice. Responses to DEP-OE were abolished in TRPA1$^{-/-}$ mice, but were unaltered by TRPV1$^{-/-}$ or TRPV$^{-/-}$ mice. Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05), calculated using an unpaired t-test.*

5.3.3 Airway Single Fibre recordings

5.3.3.1 Effect of TRPA1 antagonist on DEP-OE firing in vivo

While the isolated vagus nerve technique allows for rapid pharmacological investigation, *in vivo* investigations must still be carried out to understand the true physiological relevance of any results obtained. The effect of TRPA1 antagonism was studied *in vivo* (Figure 5.8). Firstly, DEP-OE (1µg/ml in saline, aerosolised for 60 secs) evoked action potential firing of C-Fibres in the guinea pig in a similar manner to the activation evoked by DEP in Chapter 3. Secondly, following systemic administration of Jan 130 (300mg/kg, i.p for one hour, DEP-OE failed to evoke action potential firing. Responses to acrolein (10mM, aerosolised for 60 secs) were also abolished following Jan 130, while capsaicin (100µM, aerosolised for 60 seconds) responses remained unaffected. In comparison, treatment with vehicle (0.5% methyl cellulose and 0.2% Tween in saline), did not alter DEP-OE or acrolein evoked action potential firing and capsaicin responses were also unaffected. These results demonstrate that the TRPA1 channel on airway sensory nerves plays a critical role in mediating responses to DEP-OE *in vivo.*

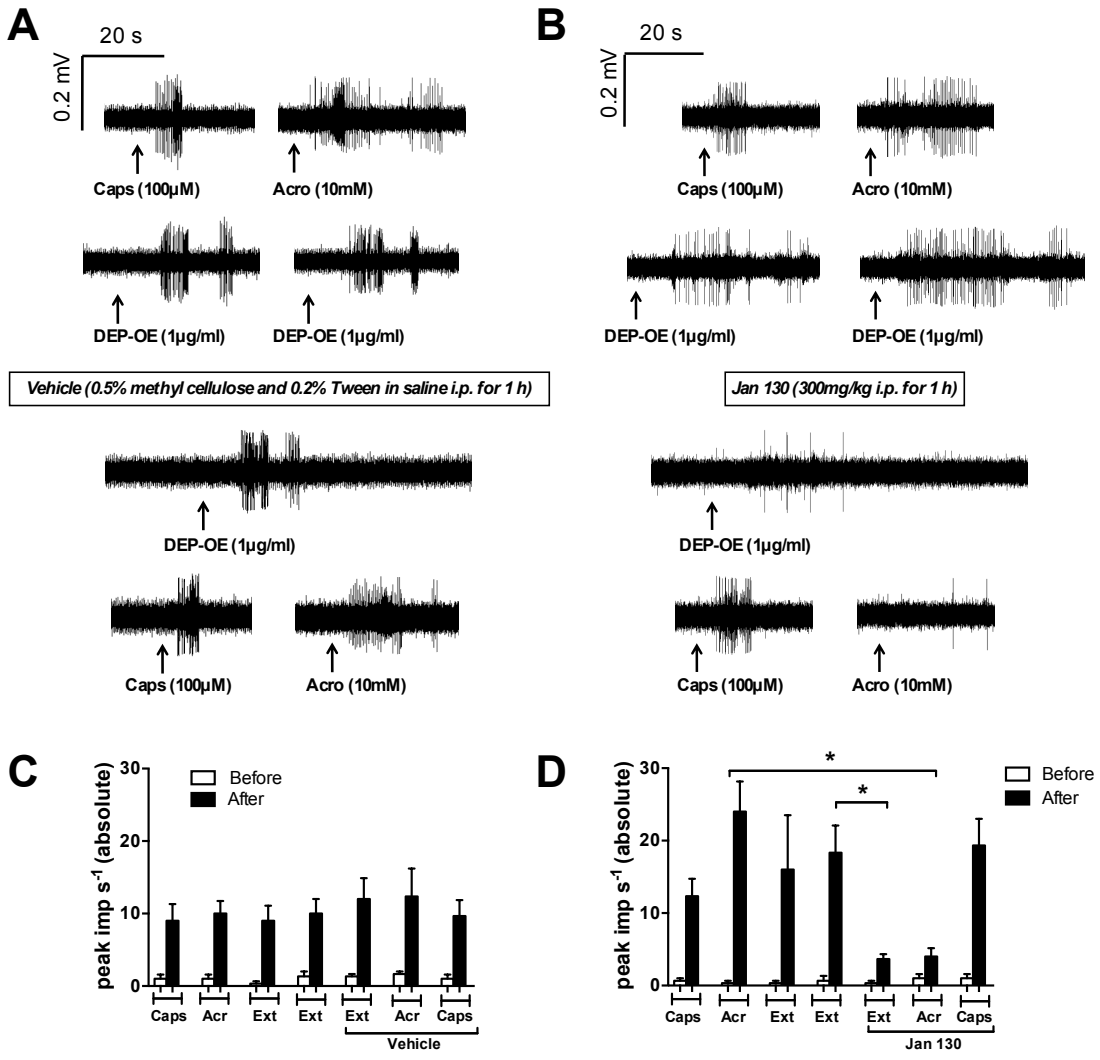


Figure 5.7: Effect of TRPA1 antagonist Jan 130 on DEP-OE induced firing of airway C-fibres.

*Positive controls of capsaicin (100µM – aerosol) and acrolein (10mM – aerosol) were used to identify the nerve fibre subtype, after which two repeatable responses to DEP-OE (1µg/ml – aerosol) were obtained prior to treatment with either vehicle (0.1% DMSO) or Jan 130 (300mg/kg) (A) Example trace shown of vehicle (0.5% methyl cellulose and 0.2% Tween in saline) treated guinea pig (B) Example trace of Jan 130 (300mg/kg i.p) treated guinea pig. Following treatment for 1 hour, the response to DEP-OE and acrolein (C) remained intact in vehicle treated animals (n=3) and (D) were significantly reduced in Jan 130 treated animals (n=3). Responses to capsaicin were unaffected by vehicle treatment or Jan 130 treatment. Data is expressed at mean ± SEM * denotes statistical significance ($P < 0.05$), calculated using a paired t-test.*

5.4 Discussion

The aim of this chapter was to investigate whether TRP ion channels played a role in the activation of airway sensory nerves by DEP-OE. TRP channels can be activated by a wide range of both endogenous and exogenous stimuli. Activation of TRP channels on airway sensory nerves is well known to trigger a variety of respiratory reflexes, resulting in respiratory symptoms such as cough and bronchoconstriction (Nasra & Belvisi, 2009; Bonvini *et al.*, 2015). Given their role in detecting and responding to environmental stimuli, it is possible that the respiratory symptoms that are associated with exposure to DEP may be due to the activation of TRP channels. At this present moment in time, there have only been a handful of papers that have specifically looked at the ability of TRP channels to be activated by DEP, despite it being one of the most prominent air pollutants in the urban environment (Shah *et al.*, 2004). TRPV1 has been implicated in the DEP induced oedema in the rat airways (Teles *et al.*, 2009). DEP has been shown to cause Ca^{2+} influx via TRPV4 in human bronchial epithelial cells (Li et al., 2011). DEP also similarly activated TRPA1 in murine DRG cells(Deering-Rice *et al.*, 2015). While these studies are helpful as a starting point, from an airway sensory nerve perspective, the cells investigated were either non-neuronal cells or not airway innervating neuronal cells.

Therefore, the aim of this chapter was to determine whether any of the same set of TRP channels (TRPA1, TRPV1 and TRPV4) were responsible for the DEP-OE induced activation of the vagus nerve that had previously been observed in Chapter 4. This was to be carried out using selective TRP channel antagonists as pharmacological tools, as well as vagal tissue from genetically modified mice.

It was confirmed that the TRP channel targets of interested were expressed at a mRNA level. Preliminary investigations detected TRPA1, TRPV1 and TRPV4 mRNA expression in whole nodose and jugular ganglia. However, the whole ganglia are made up not only of neurons, some of which innervate the airways while others innervate other organs, but cell types such as immune cells (Goehler *et al.*, 1999). As a more refined and relevant technique, single cell quantitative RT-PCR confirmed the presence of TRPA1, TRPV1 and TRPV4 mRNA in individual airway innervating neurons originating from both the nodose and jugular ganglia, which was in agreement with previous studies (Nassenstein *et al.*, 2008; Lieu *et al.*, 2012; Bonvini *et al.*, 2016). TRPV4 was only found present in one cell in the nodose sample. Discrepancies between the functional effects of TRPV4 as well as the mRNA expression levels differing between whole ganglia and individual neurons has been attributed by a secondary messenger signalling pathway involving ATP release following TRPV4 activation

(Bonvini *et al.*, 2016). One caveat of interpreting mRNA expression alone is that expression on an mRNA level does not necessarily translate into a functional protein expressed in the cell (Gygi *et al.*, 1999). For protein levels to be determined quantitatively, alternative techniques such as western blotting or immunohistochemistry must be utilised, utilising antibodies against the protein of interest. Unfortunately, research-quality antibodies for TRP channels are yet to be produced, due to the issues with antibody specificity (Saper & Sawchenko, 2003), although some papers do report TRP channel protein expression (Anand *et al.*, 2008; Yamada *et al.*, 2009). Until such a time, TRP channel mRNA expression measurement must therefore be used as a 'best attempt' technique to determine whether targets of interest are relevant in any models under investigation. However, mRNA expression can be paired with additional evidence such as the functional activity of selective TRP channel agonists to form a more robust argument that TRP channels are expressed and functional. In the case of airway sensory nerves, it has been well established that stimulation with TRPA1, TRPV1 and TRPV4 agonists have all shown to activate airway sensory nerves and to invoke airway reflexes both *in vitro* and *in vivo* (Adcock, 2009; Birrell *et al.*, 2009; Grace *et al.*, 2012; Bonvini *et al.*, 2016).

In order to probe any potential role that these TRP channels play in DEP-OE induced responses, the effects of three selective TRP channel antagonist were investigated. For TRPA1, Jan 130 was the selected antagonist (Berthelot *et al.*, 2013). For TRPV1, Xention was the selected antagonist (Round *et al.*, 2011). For TRPV4, GSK219 was the selected antagonist (Bonvini *et al.*, 2016). Initial investigations using these antagonists were carried out in the isolated vagus nerve, as this technique is more amenable to pharmacological investigations compared to the higher cost and lower throughput of *in vivo* techniques. A concentration response of these selective antagonists was carried out against their respective agonists in the isolated vagus nerve to determine appropriate concentrations to use for subsequent investigations with DEP-OE.

The application of the TRPA1 antagonist (Jan 130 - 10μM) significantly inhibited the depolarisation induced by DEP-OE in the isolated vagus nerve, while treatment with vehicle, TRPV1 antagonist (Xen – 100nM) and TRPV4 antagonist (GSK219 – 10μM) had no effect. Interestingly, the inhibitory effect of Jan 130 against whole DEP was reduced compared to the more complete inhibition of DEP-OE. With a large surface area, nanoparticles have been demonstrated to bind components of assays and interfere with the accuracy of results (Ong *et al.*, 2014), it is possible that the presence of DEP in solution was capable of binding with Jan 130, reducing its ability to successfully inhibit TRPA1. Given that DEP-OE was identified as the active component of DEP, as well as the disadvantages of DEP in terms of being less responsive to pharmacological blockade, it was decided that all subsequent investigations

would focus on DEP-OE. One of the major advantages of the *in vitro* isolated vagus nerve technique is that key experiments can be repeated in human donor tissue. DEP-OE was shown to induce depolarisation in the human vagus nerve that was significantly inhibited by Jan 130, confirming that the results obtained in the guinea pig animal model could be translated to human models.

Isolated vagal nerve tissue from genetically modified mice were utilised as an additional tool to probe the role of TRP channels in DEP-OE induced neuronal activation. The use of murine models to study airway sensory nerve physiology and their related respiratory reflexes has some limitations (Belvisi & Bolser, 2002). Specifically, mice do not have a typical cough reflex. On a broader sense, genetic KOs can also be problematic from a general research standpoint, as off target effects or functional redundancy can mean that the resultant phenotype of a mouse does not match expectations (Gingrich & Hen, 2000). Despite these limitations, genetically modified mice can still be valuable tools for researchers interested in specific cellular mechanisms, especially when suitable pharmacological tools may be lacking. Afferent limb of the reflex can be utilised as responses are similar across species including guinea pig and human.

In this chapter, firstly the genetic knock-out status of the mice was confirmed by carried out by genotyping. $TRP^{-/-}$ mice expressed a gel band product at the expected weights as compared to the WT controls. Vagal tissue from $TRP^{-/-}$ mice where then challenged with TRP agonists as a functional assessment to ensure that. As expected, vagal tissue from TRPA1 KO mice did not respond to acrolein, TRPV1 mice did not respond to capsaicin and TRPV4 mice did not respond to GSK101. With the genotype and phenotype of the KO mice confirmed, isolated vagus nerve tissue from TRP KO mice was then challenged with DEP-OE. Mirroring the pharmacological data, the depolarisation measured in response to stimulation with DEP-OE was significantly reduced in TRPA1 KO mice compared to WT control, while TRPV1 and TRPV4 mice showed no changes.

These *in vitro* studies identified a role for TRPA1 in mediating responses to DEP-OE in vagal tissues. As a technique, the *in vitro* isolated vagus nerve preparation is amenable to pharmacology investigations as it is a comparatively high throughput technique that lowers the costs of carrying out equivalent studies using *in vivo* experiments. However, the vagus nerve can innervate multiple organs within the body (Rocca & Brubaker, 1999; Brack *et al.*, 2004). As such it is unknown whether the responses obtained from the vagus nerve trunk are representative of the sensory nerves that specifically innervate the airways. Therefore, the effect of TRPA1 antagonism was studied in airway sensory nerves *in vivo*. As C-fibres were

the only fibre types that were responsive to DEP from the initial experiments in Chapter 3, the effect of TRPA1 antagonism was only studied in C-fibres challenged with DEP-OE. It was shown that DEP-OE was able to induce action potential firing in C-fibres, similarly to the whole DEP as shown in Chapter 3. Importantly, the DEP-OE induced action potential firing was significantly inhibited by the administration of a selective TRPA1 antagonist.

The *in vitro* and *in vivo* results together demonstrate a novel role for TRPA1 in mediating neuronal responses to the organic components of DEP in the airways, both in guinea pig and human vagal tissue. The current understanding of TRPA1 is that it functions as a noxious stimuli sensor (Bautista *et al.*, 2005; 2006; Kang *et al.*, 2010) (McNamara *et al.*, 2007). It can be activated by a diverse range of agonists including extracts from pungent foods such as mustard oil, garlic, wasabi and cinnamon (Macpherson *et al.*, 2007). TRPA1 has also been shown to be sensitive to environmental irritants such as acrolein that is found in cigarette smoke and other pollutants (Grace & Belvisi, 2011). TRPA1 is also thought of as an 'oxidant sensor' and can be activated by products of oxidation and reactive oxygen species (Bessac *et al.*, 2008; Liu & Ji, 2012; Kim & Hwang, 2013) . The ability of TRPA1 to be activated by such a diverse range of compounds is due to the acute sensitivity of certain cysteine residues, present on the channel's N-terminus ankyrin domains, to covalent modification by electrophilic species (Macpherson *et al.*, 2007; Takahashi *et al.*, 2008; Sadofsky *et al.*, 2011). Thus, although TRPA1 agonists may have significant differences between their chemical structures, they all possess the ability to covalently modify the channel to induce activation. TRPA1 is also reported to display sensitivity to changes in temperatures, with activation reported below 10°C (Karashima *et al.*, 2009; del Camino *et al.*, 2010).

TRPA1 agonists selectively activate C-fibres in rodents (Bessac *et al.*, 2008; Birrell *et al.*, 2009), which is in agreement with the current findings that in guinea pigs DEP-OE only activated C-fibres *in vivo.* Activation of TRPA1 on airway sensory nerves has been shown to trigger cough in both guinea pig and human subjects (Birrell *et al.*, 2009). TRPA1 has also been shown to play a role in respiratory diseases, with the activation of TRPA1 being implicated the late asthmatic response (Raemdonck *et al.*, 2012). Interestingly, asthmatics have been shown to be more sensitive to the effects of DEP exposure than healthy individuals (Nordenhäll *et al.*, 2001; Stenfors *et al.*, 2004; Mamessier *et al.*, 2006; Carlsten *et al.*, 2016). Whether the ability of DEP-OE to activate TRPA1 can explain the susceptibility of asthmatics to DEP exposure will be an important topic of research in the future.

Taken together with the established literature surrounding the airway sensory nerves and TRPA1, the results of this current chapter provide a novel neuronal mechanism to link the

exposure to DEP with respiratory symptoms. TRPA1 was identified as the key ion channel mediating the activation of airway sensory nerves by DEP-OE. However, given the complex composition of DEP and DEP-OE, further mechanistic investigations are necessary. Determining the precise agents responsible for TRPA1 activation and the underlying cellular mechanisms involved in this process will be important in advancing the knowledge of the hazards of DEP exposure.

6 Chapter Six: Investigating the mechanisms underlying DEP-OE induced activation of TRPA1

6.1 Rationale

In the previous chapter, it was demonstrated that the activation of sensory nerves by DEP-OE was mediated by TRPA1. While this result provides novel insights into the respiratory reflexes that may occur upon exposure to DEP, the mechanism underlying the activation of TRPA1 by DEP-OE is still unclear. Additionally, the key agents that are responsible for this observed activation remain to be elucidated. The organic components of DEP can vary based on a multitude of factors including fuel type and engine conditions (Geller *et al.*, 2006; Keskinen & Rönkkö, 2010). Therefore, identification of key components within DEP-OE and the mechanism by which they activate TRPA1 will allow for predictions with regard to neuronal activation to be made for other samples of DEP and PM once their chemical composition is known.

TRPA1 can either be activated by cold temperature (Kwan *et al.*, 2006; Karashima *et al.*, 2009), or by covalent modification of cysteine residues present on the channel's N-terminus (Macpherson *et al.*, 2007; Wang *et al.*, 2012). Numerous exogenous compounds possess the electrophilic properties necessary to modify TRPA1's cysteine residues, such as acrolein or cinnamaldehyde. Endogenous activators of TRPA1 include reactive oxygen species (ROS), which are highly reactive compounds that are formed during periods of oxidative stress. Such endogenous ROS include hydrogen peroxide (H_2O_2), 4-hydroxyhexanal (4-HNE) and other related lipid peroxidation products (Andersson *et al.*, 2008). TRPA1 has often been labelled as an 'oxidant stress sensor' (Bang & Hwang, 2009; Kim & Hwang, 2013) and has been shown to be activated by oxidant stress in various models, including in neuronal systems (Guidetti *et al.*, 2001; Bessac *et al.*, 2008; Taylor-Clark & Undem, 2010).

DEP has been shown to generate oxidative stress and related ROS (Hartz *et al.*, 2008; Li *et al.*, 2009b). In the lung, oxidative stress has been implicated in the progression respiratory disease states, such as lung cancer. The organic components of DEP appear to be that are responsible for oxidative stress generation (Baulig *et al.*, 2003a). Several studies have shown

that treatment with an antioxidant, such as N-acetylcysteine (NAC), can ameliorate the oxidative stress mediated effects of the organic components of DEP (Banerjee *et al.*, 2009; Carlsten *et al.*, 2014; Tseng *et al.*, 2015). DEP-OE is composed of a range of organic compounds, the majority of which are polycyclic aromatic hydrocarbons (PAHs) and their derivatives. PAHs have been identified as hazardous and toxic, posing significant risks both to human health and the environment (Ramdahl *et al.*, 1983; Kim *et al.*, 2013). While PAHs have not been shown to directly activate sensory nerves, exposure to PAHs has been shown to generate oxidative stress (Patri *et al.*, 2009). PAHs therefore may be the key component capable of activating TRPA1.

6.1.1 Chapter Hypothesis:

DEP-OE is capable of activating TRPA1 on airway sensory nerves via the generation of oxidative stress, and that this mechanism is mediated by PAHs.

6.1.2 Aims:

- To determine whether oxidative stress is capable of activating the isolated vagus nerve.
- To investigate whether oxidative stress responses are mediated by TRPA1 and that oxidative stress responses can be inhibited by antioxidants.
- To determine whether activation of the vagus nerve induced by DEP-OE can be inhibited by antioxidants.
- To determine whether PAHs are capable of activating isolated vagus nerve, and whether the mechanism of any potential activation match those identified for DEP-OE.

6.2 Methods

6.2.1 Isolated vagal nerve recordings

Vagus nerve isolated from male Dunkin Hartley guinea pigs, WT C57Bl/6 mice, $AhR^{-/-}$ KO mice or human en bloc lung tissue (unsuitable for transplant). Vagal nerve depolarisation was recorded as previously described in Chapter 2.

6.2.1.1 Pharmacological investigation into the role of oxidant stress and antioxidants on isolated vagus nerve depolarisation.

In order to determine the role that oxidative stress may play in DEP-OE induced responses, it was first necessary to confirm that oxidant stress itself was capable of activating the vagus nerve. H_2O_2 was selected as an oxidant stressor which has been shown to activate TRPA1 (Sawada *et al.*, 2008a). A concentration response was then carried out by applying non-cumulative concentrations of either vehicle (Krebs) or H_20_2 (0.01 mM to 100mM) in a randomised manner to the isolated vagus nerve. Following each two minute stimulation, the system was washed out with Krebs solution until response returned to baseline, after which the nerve was challenge with the next concentration tested to be. A submaximal concentration of H_2O_2 (10mM) was selected for subsequent antagonist studies.

After demonstrating that oxidant stress could induce depolarization in the isolated vagus nerve, antagonist studies were then carried out to confirm that the H_2O_2 response was mediated by TRPA1 using the selective TRPA1 antagonist Jan 130 (10μM). Additionally, the ability of antioxidants NAC (1mM) and reduced glutathione (GSH; 400μM) to inhibit agonist responses was also investigated.

The above antagonist studies were carried out using the standard protocol. Agonists were applied to the vagus nerve for 2 minutes to produce a control response before washing with Krebs until reaching baseline. This agonist stimulation was repeated a second time to get a reproducible response. Next, the nerve was treated with the antagonist of interest for ten minutes. Then the nerve was again challenged with the agonist in the presence of the antagonist and the resulting depolarisation recorded. This antagonist treated response was then compared to the average of the control responses. Finally, the system was washed out and the nerve challenged with the agonist a final time to generate a recovery response to confirm nerve viability. If nerves did not respond at the end of the experiment, then the result was discarded. Vehicle (0.1% DMSO for Jan 130 and Krebs solution for NAC and GSH) was also tested to confirm it did not have an effect on TRP agonist responses.

6.2.1.2 Role of the PAHs and the aryl hydrocarbon receptor in isolated vagus nerve depolarisation.

To identify which specific chemicals present in DEP-OE were responsible for the observed activation of the isolated vagus nerve, the certificate of analysis for SRM-1975 was consulted

(See Table 6.1 for a selection of concentration values). Of the dozens of PAHs present in SRM-1975, phenanthrene was selected due to its relatively high concentration in SRM-1975, its presence in other DEP samples (Bayram *et al.*, 2009) and having been shown to a critical component of DEP that enhances IgE production *in vitro* (Tsien *et al.*, 1997).

Table 6.1: List of selected PAHs present in SRM-1975 and their mass fractions *(Source – Certificate of Analysis for SRM-1975, NIST, USA).*

	Mass Fractions (mg/kg)[a]
Phenanthrene[b,c]	8.00 ± 0.20
Fluoranthene[b,c]	13.5 ± 0.6
Benz[*a*]anthracene[b,d,e]	0.092 ± 0.015
Chrysene[c,d,e]	1.95 ± 0.07
Triphenylene[c,d,e]	2.38 ± 0.10
Benzo[*b*]fluoranthene[c,d,e]	3.20 ± 0.10
Benzo[*k*]fluoranthene[c,d]	0.174 ± 0.050
Benzo[*e*]pyrene[b,d]	0.268 ± 0.023

A concentration response was then carried out by applying non-cumulative concentrations of either vehicle (0.1% DMSO) or phenanthrene (10^{-12}M to 10^{-8}M) in a randomised manner to the isolated vagus nerve. Following each two minute stimulation, the system was washed with Krebs solution until the response returned to baseline, after which the nerve was challenge with the next concentration was tested. From these studies, an appropriate concentration of phenanthrene (10^{-9} M) was selected to be used for subsequent antagonist studies.

To investigate whether phenanthrene activated TRPA1 in a similar manner to DEP-OE, a pharmacological approach was used. The effect of either vehicle (0.1% DMSO) or Jan 130 (10μM) on phenanthrene (10^{-9} M) induced depolarisation was carried out using the standard antagonist protocol as described above.

Phenanthrene, like other PAHs, is thought to primarily exert its toxic and carcinogenic effects through the AhR (Rowlands & Gustafsson, 1997; Denison *et al.*, 2011). CH-223191 (10μM (Zhao *et al.*, 2010)) and 6,2',4'-trimethoxyflavone (TMF; 10μM (Murray *et al.*, 2010)) were chosen as two selective AhR antagonists to determine the role of AhR in phenanthrene (10^{-9} M) induced responses, as well as DEP-OE (1μg/ml) induced responses. The effect of AhR antagonists against TRP channel agonists capsaicin (1μM) and acrolein (300μM) induced responses was also investigated.

6.2.1.3 Effect of TRP agonists and DEP-OE in genetically modified mouse vagal tissue

To complement the data generated from pharmacological studies, vagal tissue from WT (C57Bl/6) and $AhR^{-/-}$ mice was challenged with 2 minute stimulations of capsaicin (1μM), acrolein (300μM) and DEP-OE (1μg/ml) in a randomized manner. Following stimulation, the system was washed with Krebs to baseline, then the nerve challenged with the next stimulation.

6.2.1.4 Pharmacological investigation into the role of mitochondrial oxidative stress in DEP-OE induced responses.

To determine whether mitochondrial oxidative stress was capable of depolarizing the vagus nerve, the effect of antimycin A was investigated. Antimycin A is an inhibitor of the electron transport chain within the mitochondria, leading to incomplete reduction of O_2 and the generation of superoxides (St-Pierre *et al.*, 2002). Although antimycin A irreversibly binds to the mitochondrial complex III Q_i site (Rieske *et al.*, 1967), a concentration response was carried out in a randomised manner as described above. From the results of the concentration response, 20μM was selected as a dose for antagonist studies.

MitoTEMPO, a mitochondrial specific superoxide scavenger, has been shown to inhibit neuronal activation induced by mitochondrial ROS (Nesuashvili *et al.*, 2013a). The effect of MitoTEMPO on antimycin A (20μM), acrolein (300μM) and DEP-OE (1μg/ml) was investigated. Given the irreversible binding nature of antimycin A, the standard antagonist protocol could not be followed as it would have required multiple stimulations of antimycin A. Instead, an alternative antagonist protocol was followed. Firstly, the nerve was challenged with a 2 minute stimulation with acrolein (300μM) to confirm nerve viability. Then the nerve was incubated for 10 minutes with either vehicle (0.1% DMSO) or MitoTEMPO (2μM) before a 2 minute stimulation with the agonist of interest. After washout with Krebs, the nerve was challenged for a final time with acrolein to confirm nerve viability at the end of the experiment.

6.2.1.5 Analysis

The chart recorder was calibrated so that 1mm was equal to 0.01mV. Vagal nerve depolarisation was measured in mV as read from the chart recorder graph paper. For antagonist studies following the regular protocol, depolarisations that occurred in the presence of antagonist drugs were compared against the average depolarisation of the prior control

responses in the same tissue to calculate antagonist % inhibition. Statistical calculations of these antagonist effects were calculated using a paired two tailed t-test comparing the antagonist responses to the control responses within the same nerve. For the alternative antagonist profile, an unpaired T-test was used to compare the agonist responses after incubation with either vehicle or MitoTEMPO in different pieces of vagal nerve. For experiments utilising $AhR^{-/-}$ vagal tissue, depolarisations in WT controls were compared to those from $AhR^{-/-}$ tissue using an unpaired t-test. Data is presented as mean ± S.E.M, with statistical significance set at $P < 0.05$.

6.3 Results

6.3.1 H_2O_2 induced activation of vagus nerve

H_2O_2 depolarized the guinea pig isolated vagus nerve in a concentration depended manner. H_2O_2 (100mM) induced the maximum depolarization of 0.155 mV ± 0.02. A sub-maximal concentration of 10mM was selected for subsequent antagonist studies (Fig 6.1).

In the guinea pig vagus nerve, application of the selective TRPA1 antagonist Jan 130 (10μM) significantly inhibited the response to H_2O_2 (10mM). The selective TRPV1 antagonist Xention (100nM) and vehicle (0.1% DMSO) had no significant effect on the vagal depolarisation induced by H_2O_2 (Fig 6.2a). Repeating this key experiment in human vagal tissue, preliminary results indicated that Jan 130 had a similar effect on H_2O_2 (10mM) induced depolarisation (Fig 6.2b).

6.3.2 Effect of antioxidants on DEP-OE induced depolarisation of vagus nerve

In the isolated guinea pig vagus nerve, application of NAC (1mM) inhibited responses to H_2O_2 (10mM) by 97.7% ± 2.3. An alternative antioxidant GSH (300μM) also inhibited responses to H_2O_2 (10mM) by 86.3% ± 13.6 (Fig 6.3A/B). In human vagal tissue, preliminary results indicated that NAC (1mM) also inhibited responses to H_2O_2 (10mM) (Fig 6.3C).

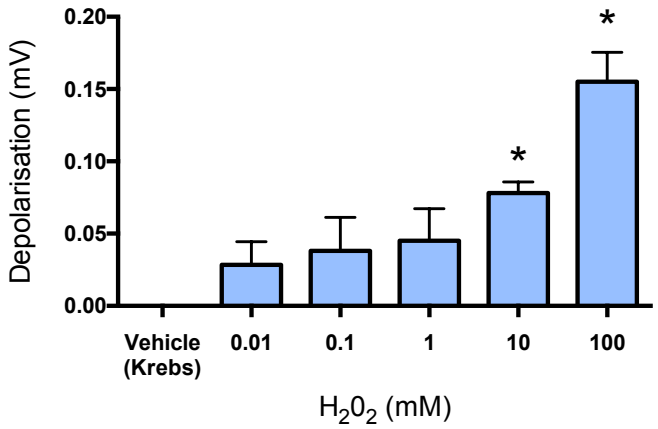


Figure 6.1: Concentration response curves of H_2O_2 on isolated vagal nerve depolarisation.

*H_2O_2 induced a concentration dependent depolarisation of the isolated guinea pig vagus nerve (n=4-6). A submaximal concentration (10mM) was selected as the concentration to continue further pharmacological investigations. Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05), calculated using a Kruskal-Wallis and Dunn's post-hoc test comparing against vehicle.*

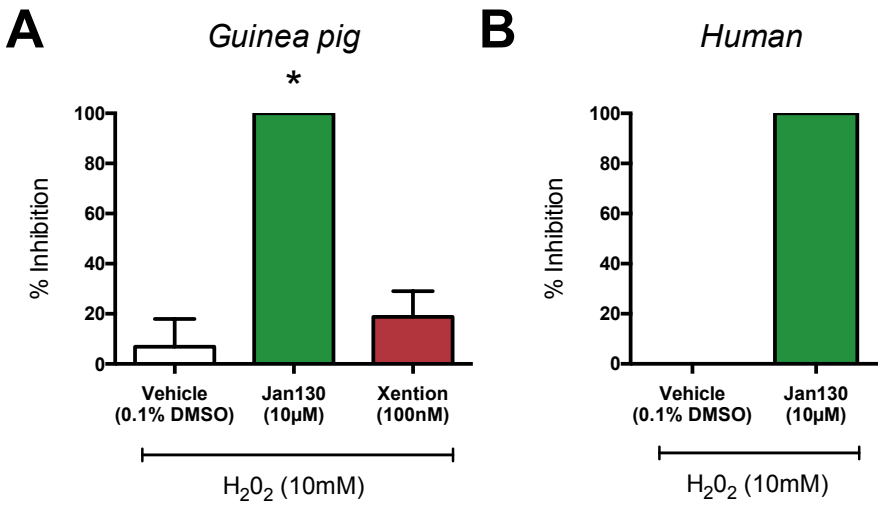


Figure 6.2: Effect of selective TRP antagonists on H_2O_2 induced depolarisation

*Selective TRP channel antagonists were used to investigate the role of TRP channels in the depolarisation induced by H_2O_2. **(A)** The selective TRPA1 antagonist Jan 130 (10μM) completely inhibited the response to H_2O_2 (10mM), while Xention (100nM) had no significant effect (n=4-5). **(B)** Jan 130 also completely inhibited responses to H_2O_2 in human tissue (n=2). Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05) comparing antagonist treatment responses with control responses in the same tissue as calculated using a paired t-test.*

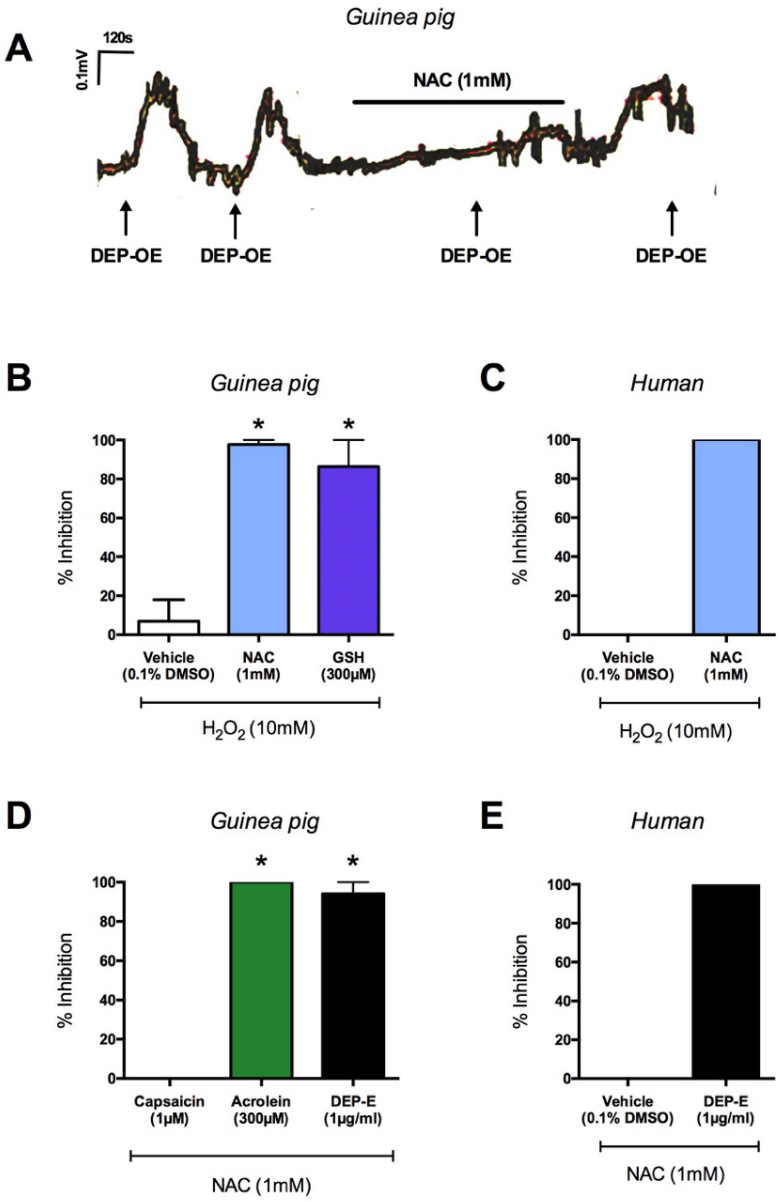


Figure 6.3: Effect of NAC on oxidative stimuli, TRP agonists and DEP-OE

(*A*) *Representative trace showing the inhibition of H_2O_2 induced depolarisation in the guinea pig vagus nerve.* ***(B)*** *Histogram showing that NAC (1µM) and GSH (300µM) were able to significantly inhibit responses to H_2O_2 (10mM) (n=4-5)* ***(C)*** *NAC (1µM) inhibited response to H_2O_2 (10mM) in human vagal tissue. (n=2)* ***(D)*** *NAC (1µM) inhibited responses to DEP-OE (1µg/ml), as well as the TRPA1 agonists acrolein (300µM) but had no effect on capsaicin (1µM) responses. (n=4)* ***(E)*** *NAC (1µM) inhibited response to DEP-OE (1µg/ml) in human vagal tissue (n=2). Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05) comparing antagonist treatment responses with control responses in the same tissue as calculated using a paired t-test.*

The effect of NAC (1mM) on TRP agonists and DEP-OE responses was then investigated. In the isolated guinea pig vagus nerve, NAC (1mM) had no effect on capsaicin (1μM) induced responses, but abolished acrolein (300μM) responses and inhibited DEP-OE (1μg/ml) responses by 94.1% ± 5.9 (Fig 6.3D). In human tissue, preliminary results indicated that NAC (1mM) also completely inhibited responses to DEP-OE (1μg/ml) (Fig 6.3E).

6.3.3 Role of Aryl Hydrocarbon Receptor

Phenanthrene (a classical PAH) was identified as a major component of DEP-OE. Phenanthrene depolarised the isolated vagus nerve by 0.09 mV ± 0.006 (Fig 6.4)

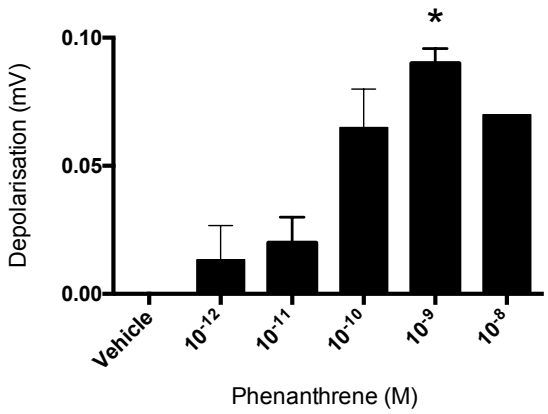


Figure 6.4: Investigating the effect of phenanthrene on vagal depolarization

*Phenanthrene induced a concentration dependent depolarization of the isolated guinea pig vagus nerve (n=2-3). SEM * denotes statistical significance (P < 0.05) Data is expressed at mean ± SEM*

PAHs such as phenanthrene traditionally signal through the AhR. Two selective AhR antagonists, CH-223191 (10μM) and TMF (10μM) both significantly inhibited responses to phenanthrene (10^{-9}M) by 94.4% ± 5.6 and 65 ± 23.6 respectively. To confirm that phenanthrene was activating TRPA1 in a similar manner to DEP-OE, the effect of Jan 130 (10μM) on phenanthrene induced depolarisation was investigated. Jan 130 (10μM) abolished responses to phenanthrene (10^{-9} M) (Fig 6.5A).

With the AhR antagonists confirmed to block responses to a traditional ligand in phenanthrene, their effects against TRP agonists and DEP-OE were then investigated. CH-223191 (10μM) had no effect on either capsaicin (1μM) or acrolein (300μM), but significantly inhibited responses to DEP-OE (1μg/ml) by 72.3% ± 12.0. In a similar manner, TMF had no effect on

acrolein (300μM) induced responses but inhibited DEP-OE (1μg/ml) responses by 91.0% ± 9.0 (Fig 6.5B/C).

6.3.4 Effect of TRP agonists and DEP-OE in AhR$^{-/-}$ mice.

Vagal tissue from AhR$^{-/-}$ mice and WT controls were challenged with capsaicin (1μM), acrolein (300μM) and DEP-OE (1μg/ml). Only a few AhR$^{-/-}$ mice were available but preliminary results indicated that capsaicin (1μM) and acrolein (300μM) responses were unaltered, but DEP-OE were reduced in AhR$^{-/-}$ mice compared to WT controls (Figure 6.6).

6.3.5 Role of mitochondrial oxidative stress

The effect of antimycin A, a toxin capable of generating mitochondrial superoxide, was investigated. In the isolated guinea pig vagus nerve antimycin A depolarised the vagus nerve in a concentration depended manner, with the top concentration of 20μM depolarising the nerve by 0.07 mV ± 0.03 (Fig 6.7).

As a pharmacological tool to investigate the role of mitochondrial oxidative stress, the effect of mitochondrially targeted antioxidant MitoTEMPO was investigated (Fig 6.8). MitoTEMPO (2μM) completely inhibited responses to antimycin A (20μM), but had no effect on acrolein (300μM) induced responses. The application of MitoTEMPO (2μM) significantly inhibited responses to DEP-OE (1μg/ml).

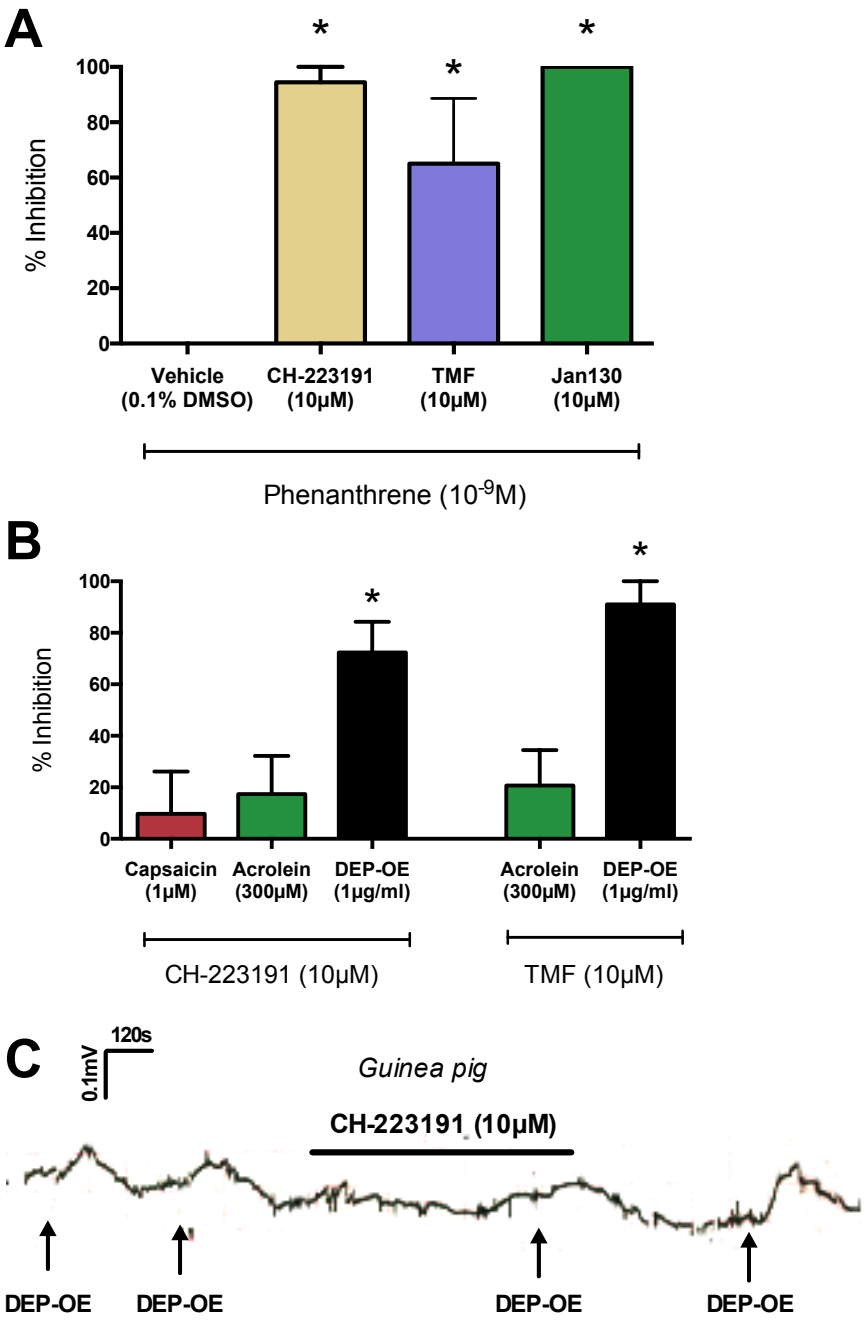


Figure 6.5: Effect of AhR antagonists on phenanthrene, TRP agonists and DEP-OE.

(A) *Depolarisation induced by phenanthrene was inhibited by two AhR antagonist, CH-223191 (10μM) and TMF (10μM), as well as the TRPA1 antagonist Jan 130 (10μM (n=3-5).* ***(B)*** *CH-223191 (10μM) and TMF (10μM) inhibited responses to DEP-OE (1μg/ml), but responses to the TRPV1 agonist capsaicin (1μM) and TRPA1 agonist acrolein (300μM) were unaffected (n=4-6).* ***(C)*** *Representative trace of CH-223191 (10μM) inhibiting responses to DEP-OE (1μg/ml) in the guinea pig vagus nerve. Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05) comparing antagonist treatment responses with control responses in the same tissue as calculated using a paired t-test.*

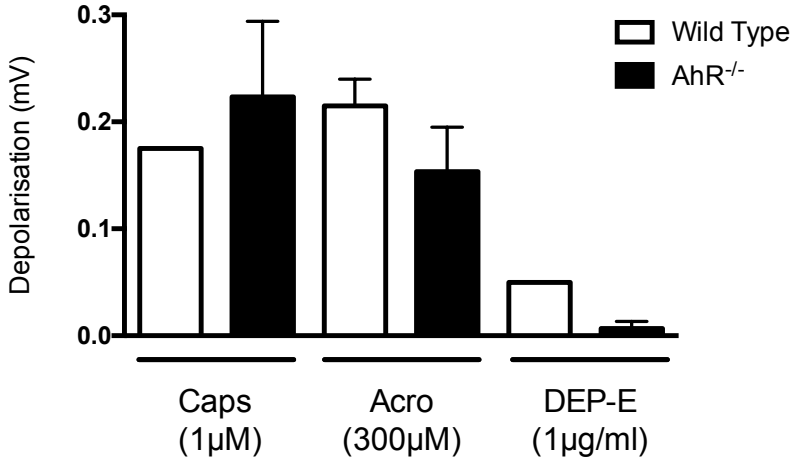


Figure 6.6: Effect of DEP-OE in TRP KO mice.

Depolarisation induced by the TRPV1 agonist capsaicin (1µM) and the TRPA1 agonist acrolein (300µM) were unaltered in AhR$^{-/-}$ mice, however preliminary results indicate that responses to DEP-OE (1µg/ml) were reduced (n=2-3). Data is expressed at mean ± SEM.

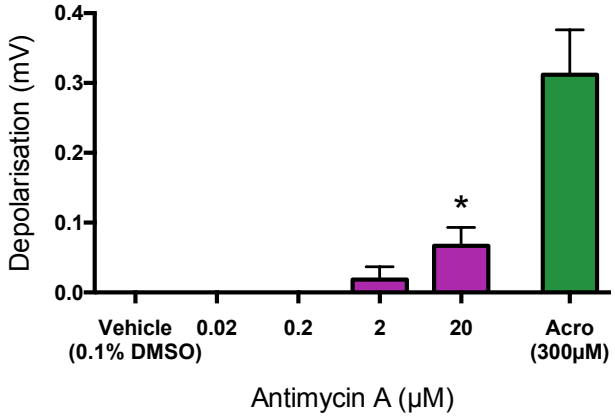


Figure 6.7: Effect of mitochondrial oxidant stress on isolated vagal nerve depolarisation.

*Antimycin A induced a concentration dependent depolarization of the isolated guinea pig vagus nerve (n=5-6). Acrolein (300µM) is shown as a positive control. 20µM was selected as the concentration to continue further pharmacological investigations. Data is expressed at mean ± SEM * denotes statistical significance (P < 0.05), calculated using a Kruskal-Wallis and Dunn's post-hoc test comparing against vehicle.*

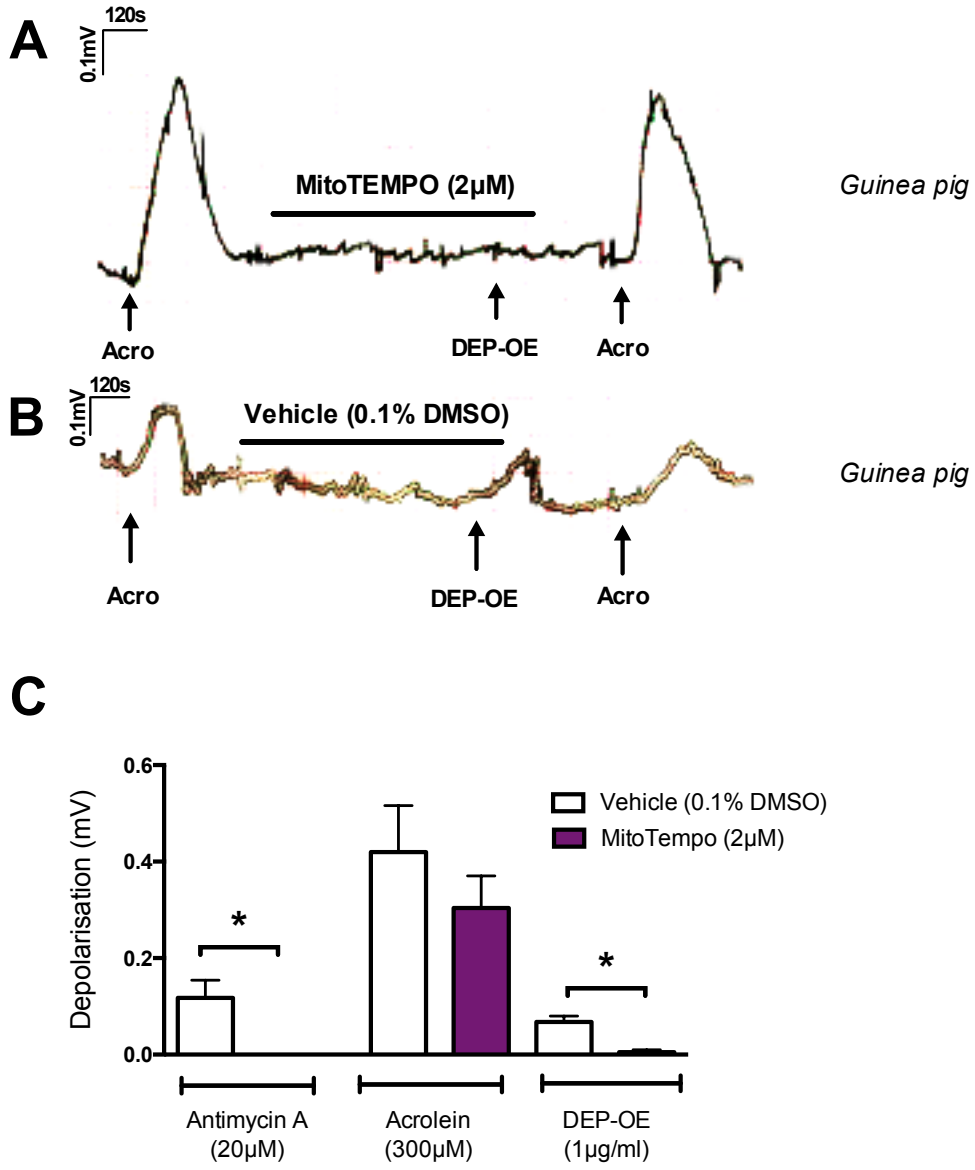


Figure 6.8: Effect of a mitochondrially targeted antioxidant on agonist responses

A) *Representative trace of the effect of the mitochondrially targeted antioxidant MitoTEMPO (2μM) on DEP-OE (1μg/ml) induced depolarisation* ***B)*** *Representative trace of the effect of vehicle (0.1% DMSO) on DEP-OE (1μg/ml) induced depolarisation* ***C)*** *Histograms showing inhibition of antimycin A (20μM) induced responses by MitoTEMPO (2μM). DEP-OE (1μg/ml) were also inhibited, while responses to the TRPA1 agonist acrolein (300μM) were unaffected. Data is expressed at mean ± SEM * denotes statistical significance ($P < 0.05$), calculated using an unpaired t-test comparing agonist responses after treatment with either vehicle or antagonists in separate pieces of nerves.*

6.4 Discussion

This chapter aimed to determine the underlying mechanisms by which DEP-OE activated TRPA1 ion channels on airway sensory nerves.

There is an increasing body of evidence demonstrating that the adverse health effects of air pollution can be attributed to its ability to generate oxidative stress (Kelly, 2003). Oxidative stress is a term to describe an abnormal redox state within the cell whereby the presence of excessive oxidants overwhelm the innate antioxidant systems within a cell (Sies, 1997). Examples of such oxidants include reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot-}$) or hydroxyl ($OH^{\cdot}$) free radicals that possess one or more unpaired electrons (thus making them highly reactive) or non-radical oxidisers such as H_2O_2 or ozone (O_3) that can be easily converted into free radicals (Bayr, 2005). ROS indiscriminately react with lipids and proteins within the cell, and thus be harmful if present in excessive amounts (Yu, 1994). Small levels of ROS are generated during normal cellular respiration, whereby the production of ATP by the mitochondrial electron transport chain require O_2 to act as an electron donor. This process results in the production of superoxides and hydrogen peroxide, which are normally detoxified by free radical scavenging enzymes or antioxidants such as GSH (Andreyev *et al.*, 2005).

By being in intimate contact with the external environment, the airways can be exposed to additional sources of oxidative stress. Aqueous extracts of DEP are capable of generating malondialdehyde from 2-deoxyribose, through the production of ROS (Ball *et al.*, 2000). DEP form superoxides that promote DNA damage in the presence of cytochrome p450 reductase (Kumagai *et al.*, 1997). Whether the generation of such oxidant stress by DEP can activate sensory nerves has not yet been investigated.

Previous studies have shown that application of H_2O_2, a classic oxidant stressor, activates abdominal C-fibres (Stahl *et al.*, 1993) and cardiac sympathetic afferents (Huang *et al.*, 1995) in feline models. Pulmonary C-fibres have also shown to be activated by oxidants (Taylor-Clark *et al.*, 2008). Initial experiments in this chapter aimed to determine whether oxidant stress was capable of activating airway sensory nerves *in vitro* using the isolated vagus nerve preparation. H_2O_2 depolarised the isolated guinea pig vagus nerve in a concentration depended manner, confirming that oxidant stress was capable of inducing responses in the *in vitro* preparation.

A submaximal dose of H_2O_2 was used for subsequent experiments to select an appropriate antioxidant to use as a tool to probe oxidative mechanisms. NAC is a thiol and a commonly used antioxidant with two mechanisms of action (Zafarullah *et al.*, 2003). Acting as a scavenger, it is capable of directly neutralising oxidative species in solution (Aruoma *et al.*, 1989) as well being a precursor for endogenous thiols within the cell such as GSH (Bridgeman *et al.*, 1991). NAC inhibited depolarisation induced by H_2O_2 in both guinea pig and human

tissue. Further experiments confirmed that NAC had no effect on responses to the TRPV1 agonist capsaicin, but also blocked responses to TRPA1 agonists acrolein, a reactive electrophilic compound. Application of GSH to increase the concentration of the endogenous antioxidant defences also inhibited responses to H_2O_2. These results demonstrated that antioxidants were capable of inhibiting responses to electrophilic specifies and oxidants, as expected. Taking NAC forward as a tool, it was then shown that NAC inhibited responses to DEP-OE both in guinea pig and human isolated vagus tissue. To summarise, these results indicate that the depolarisation induced in sensory nerves by DEP-OE is mediated by the generation of oxidative stress. The efficacy of NAC in inhibiting responses to DEP-OE are in agreement with a previous study showing that the administration of NAC to asthmatic patients attenuated the adverse respiratory effects of DEP exposure (Carlsten *et al.*, 2014).

The ability for DEP to generate oxidative stress also explains the role of TRPA1 in the observed activation of airway sensory nerves. Through covalent modifications of cysteine residues present on the cytosolic N-terminus of the ion channel, TRPA1 can be activated by a range of ligands (Wang *et al.*, 2012). Although acrolein, isothiocynates and cinnamaldehyde are all structurally distinct, they share common electrophilic chemical properties that allow such ligands to covalently activate TRPA1 (Macpherson *et al.*, 2007) In a similar manner, oxidant stressors and related ROS have been shown to activate TRPA1. Application of oxidants produced an itch indicative scratching behaviour in mice which was alleviated by the application of either NAC or trolox, a vitamin E analogue that acts as an antioxidant, or the selective TRPA1 antagonist HC-030031 (Liu & Ji, 2012). Multiple studies have demonstrated H_2O_2 induced Ca^{2+} influx via TRPA1 in murine sensory neurons (Andersson *et al.*, 2008; Bessac *et al.*, 2008; Sawada *et al.*, 2008b). It was confirmed in this present study that in a similar manner to DEP-OE, depolarisation induced by H_2O_2 was mediated by TRPA1, as application of the selective TRPA1 antagonist Jan 130 prevented depolarisation.

As the overall aim of this thesis was to understand how air pollutants such as DEP can cause adverse respiratory effects, it was also necessary to clarify which specific chemical components of DEP were responsible for generation of oxidative stress and the resulting activation of TRPA1. The results from Chapter 3 indicated that the organic compounds were the key components of DEP with regards to neuronal activation. A certified list of organic hydrocarbons present in DEP-OE can be obtained from the manufacturer (NIST; Certificate of Analysis – SRM 1975). From the list, phenanthrene can be identified as one of the most abundant hydrocarbons present. Phenanthrene is a classical PAH, a range of hydrocarbons classified as environmental pollutants that are characterised by containing fused benzene rings (Kanaly & Harayama, 2000) and are generated during the combustion of fossil fuels

(Nikolaou *et al.*, 1984). PAHs have been identified as potent toxins and carcinogens both to humans (Kim *et al.*, 2013) and wildlife, particularly marine life where PAHs can adversely affect embryonic development (Black *et al.*, 1983; Horng *et al.*, 2010). With a three ring structure, phenanthrene is one of the more simple forms of PAHs and has been shown to a key component of DEP that enhances IgE production *in vitro* (Tsien *et al.*, 1997). When compared to other organs such as the skin and bladder, the lung has been identified as one of the major target organs for PAH carcinogenicity (Boffetta *et al.*, 1997). However, the ability for sensory nerves to be directly activated by PAHs had not been investigated. Phenanthrene depolarised the vagus nerve in a concentration dependent manner, at a similar magnitude to that of DEP-OE. This activation was also shown to be mediated via TRPA1, indicating that PAHs such as phenanthrene were the critical components of DEP-OE that were responsible for the activation of airway sensory nerves.

PAHs are traditionally thought to exert their harmful effects through the activation of AhR. A wide variety of environmental pollutants, including PAHs, are ligands for AhR which is a highly conserved transcription factor (Denison & Nagy, 2003). When activated, AhR binds to xenobiotic response elements of genes to drive the transcription and translation of a variety targets related to metabolism of environmental toxins in an attempt to protect the cell (Hankinson, 1995). Examples include CYP1A1, an enzyme that is upregulated several hours after initial AhR activation and is capable of metabolising a large number of xenobiotics (Brauze *et al.*, 2006). However, metabolism of PAHs by CYP1A1 results in the formation reactive harmful intermediates that can covalently bind to DNA and proteins (P L Grover, 1968; Nebert *et al.*, 2004). PAHs can be metabolised by CYP1A1 to their highly electrophilic derivatives (Jacob *et al.*, 1996; Schober *et al.*, 2010), forms of which have been shown to activate TRPA1 (Ibarra & Blair, 2013).

Metabolic activation of PAHs by AhR signalling cascades has traditionally been measured in hours (Brauze *et al.*, 2006), rather than more immediate neuronal responses that have been observed in this chapter. Despite this, given that AhR is the primary target for PAHs, the effect of selective AhR antagonists was investigated. Application of CH-223191, a selective AhR antagonist that has been shown to competitively inhibit AhR-ligand binding without exhibiting AhR agonist-like activity (Kim *et al.*, 2006; Zhao *et al.*, 2010), inhibited depolarisation induced by phenanthrene. TMF, a second structurally unrelated specific AhR antagonist (Murray *et al.*, 2010), also inhibited the responses to phenanthrene. Both of these antagonists inhibited responses to DEP-OE, but had no effect on direct TRP agonists. These pharmacological results are in agreement with preliminary findings that the vagal tissue of $AhR^{-/-}$ mice did not

respond to DEP-OE, but responses to TRP agonists were not altered compared to WT controls.

Together, these results demonstrate that the AhR plays a key role in the rapid neuronal activation induced by PAHs, such as phenanthrene, present in DEP-OE. The overwhelming majority of existing literature supports the notion that AhR exerts its effects through altering gene expression (Rowlands & Gustafsson, 1997; Lin *et al.*, 2003; Brauze *et al.*, 2006). These present results provide evidence for a non-canonical role for AhR, mediating the immediate activation of TRPA1. However, there is some evidence that AhR may be involved in cellular processes that do not involve the traditional gene transcription and translation pathways. Cross talk between AhR and various protein kinases and NF-κB signalling pathways have been identified, although the underlying mechanisms behind such cross talk remains to be elucidated (Denison *et al.*, 2011).

Interestingly, AhR has been associated with mitochondrial ROS production via mechanisms that are independent of CYP1A1, (Senft *et al.*, 2002). Antimycin A is an inhibitor of the electron transport chain at the mitochondrial complex III Q_i site that results in a blockade of electron flow, which leads to incomplete reductions of O_2 and the generation of superoxides (St-Pierre *et al.*, 2002). By generating mitochondrial ROS, antimycin A has been shown to activate bronchopulmonary C-fibres in mice and this activation was mediated by TRPA1 (Nesuashvili *et al.*, 2013b). In this present study, antimycin A depolarised the isolated vagus nerve in a concentration dependent manner. MitoTEMPO, a mitochondrially targeted superoxide scavenger, inhibited depolarisation induced by antimycin A. MitoTEMPO had no effect on responses to acrolein, unlike NAC, demonstrating that its effects were localised to the mitochondria. MitoTEMPO inhibited responses to DEP-OE, indicating that mitochondrial ROS played a critical role in the observed activation of the vagus nerve by the organic components of DEP.

The results of this chapter highlight a novel mechanism by which air pollutants such as DEP activate airway sensory nerves, and in doing so have revealed a non-classical role for AhR. Having identified a mechanistic pathway, the potential impacts of a variety of air pollutants on airway sensory nerve activation can be assessed based on their knowledge of their chemical composition. The wider implications of how these results can further knowledge of the health effects of air pollution will be discussed in the next chapter.

7 Chapter Seven: Conclusions

7.1 Thesis summary

It is now accepted that exposure to urban air pollution is associated with a range of adverse health effects (Brunekreef & Holgate, 2002; Kelly & Fussell, 2011; Maynard, 2015). Air pollution is composed of gases and PM suspended within the air (Kelly & Fussell, 2015) and studies have highlighted that many of the effects of wider air pollution can be correlated with PM exposure (Zanobetti & Schwartz, 2006; Kang *et al.*, 2016). However, discrepancies are found in the literature as to how PM exposure can affect health. Gaps in scientific knowledge are likely due to the complex composition of PM that can make it difficult to identify underlying 'cause and effect' (Delmelle *et al.*, 2002; Davidson *et al.*, 2005; Sacks *et al.*, 2011). Given that the number of individuals living in urban environments is increasing, there is a need to further explore the mechanisms by which such air pollutants cause harm so efforts can be made to avoid morbidity and mortality.

In cities, human activities such as the combustion of fossil fuels contribute significantly to total PM levels (Harrison *et al.*, 1997; Kleeman & Cass, 1998; Song *et al.*, 2001). One of the major potential sources of urban PM are the emissions of diesel engines, particularly in Europe where diesel powered vehicles are increasing in popularity (Cames & Helmers, 2013). DEP are formed from the incomplete combustion of diesel fuel (Lloyd & Cackette, 2001) and have been shown in epidemiological studies to be associated with respiratory symptoms such as cough, wheeze and reductions in lung function (Kagawa, 2002; McCreanor *et al.*, 2007; Zhang *et al.*, 2009; Bernstein, 2012; Ghio *et al.*, 2012b). These associations have also been demonstrated using controlled exposures to DEP in laboratory settings (Nightingale *et al.*, 2000; Törnqvist *et al.*, 2008; Carlsten *et al.*, 2016). The majority of research aiming to determine the mechanisms responsible for such health effects have primarily focused on oxidative stress and inflammatory mediator release in epithelial or immune cells within the lung (Ohtoshi *et al.*, 1998; Jantzen *et al.*, 2012; Schwarze *et al.*, 2013).

However, another potential mechanism by which DEP may affect the respiratory system is through the activation of airway sensory nerves. The lungs are in intimate contact with the external environment and as such the body requires the ability to detect and respond to stimuli that may damage the airways. Afferent nerves that innervate the airways possess numerous receptors on their surface such as TRP ion channels that can be activated by a range of exogenous and endogenous stimuli, including changes in temperature, acidity, osmolarity, inflammatory mediators, oxidative stress and mechanical forces (Nasra & Belvisi, 2009;

Bonvini *et al.*, 2015; Taylor-Clark, 2015). Activation of TRPs leads to an influx of Ca^{2+} and with sufficient depolarisation an action potential is generated and a signal is sent up the vagus nerve to the central nervous system (Widdicombe, 1995). After processing, a signal is sent back down efferent pathways that can trigger a range of defensive respiratory reflex responses, such as cough, mucus secretion and bronchoconstriction (Coleridge & Coleridge, 1994; Belvisi, 2002).

As airway sensory nerves are designed to protect the airways from harmful substances, it was logical to hypothesise that they may be activated by air pollutants such as DEP. Additionally, many of the symptoms that are associated with DEP exposure are the similar to those that are triggered by the activation of airway sensory nerves. Activation of airway sensory nerves may therefore be an important mechanism that drives the association between adverse respiratory effects and exposure to DEP. A handful of studies have demonstrated that DEP can activate TRP channels, including TRPA1, TRPV1 and TRPV4 (Teles *et al.*, 2009; Deering-Rice *et al.*, 2011; Li *et al.*, 2011). However, to date no studies have specifically investigated whether airway sensory nerves can be activated.

Experiments were performed in guinea pigs as they have similar vagal nerve anatomy and respiratory reflex responses to humans (Belvisi & Bolser, 2002). Initially, it was demonstrated that in the anesthetised guinea pig an intratracheal administration of DEP generated action potential firing in airway C-fibre afferents *in vivo*. Various subtypes of airway sensory nerve exist, and C-fibres commonly detect the presence of noxious chemical stimuli. However, DEP was unable to activate A-δ fibres which primarily respond to mechanical stimuli or changes in osmolarity. These results suggested that the chemicals present in DEP rather than the physical presence of the carbon core in the airways may play an important role. Due to the time and expense limitations of *in vivo* single fibre recordings, the effects of DEP were then studied *in vitro*. DEP depolarised the isolated guinea pig vagus nerve in a concentration dependent manner and this depolarisation was shown to be inhibited by the presence of the fast Na^{+} channel blocker TTX, confirming the neuronal basis of the responses observed. DEP was also shown to activate isolated human vagus nerve, highlighting that results were likely to be translatable to man. Together these first results confirmed that DEP was capable of activating airway sensory nerves both *in vivo* and *in vitro,* and interestingly this activation was mediated by chemosensitive pathways rather than mechanically sensitive ones.

DEP is highly heterogeneous in its composition (Lidia Morawska *et al.*, 1998; BeruBe *et al.*, 1999; Sharma *et al.*, 2005; Wichmann, 2008). As such, it was necessary to physicochemically characterise DEP so that the physiological results could be properly interpreted and potential

properties that may drive the activation of the vagus nerve could be identified (Warheit, 2008). Using dynamic light scattering (DLS) and cryogenic electron microscopy (cryo-EM), the average aerodynamic size of DEP was confirmed to be <1μM in size, indicating that it was easily respirable (*Aerosol Technology*, 2012). Measurements of the metal content using energy dispersive X-Ray (EDX) analysis did not find any species of note, in keeping with previous studies (Ball *et al.*, 2000; Jensen, 2006). Using thermogravimetric analysis (TGA), DEP was found to be composed of mainly of inorganic carbon (83%), with some organic hydrocarbons (15%) and the remaining trace impurities.

With negligible metal content, the role that the organic components of DEP played in the activation of airway sensory nerved was investigated. The particulate carbon core of DEP (par-DEP) was separated from the organic components (org-DEP) using Soxhlet extraction. Only org-DEP (1μg/ml), not par-DEP (1μg/ml), depolarised the isolated vagus nerve and this depolarisation was of a similar magnitude to that of whole DEP (1μg/ml). To mitigate variations that would occur using self-made extractions, commercially available organic extracts of DEP (DEP-OE) were purchased and shown to depolarise the vagus nerve in a concentration dependent manner in a similar manner to whole DEP. Carbon black, an analogue of the carbon core of DEP, had no effect. These results demonstrated that the organic compounds within DEP were the key components in terms of vagus nerve activation and indicate that the carbon core is merely acting as a carrier. These results are in agreement with the *in vivo* single fibre recordings that found that only chemosensitive airway afferents were activated by DEP. Other studies within the literature have highlighted that the organic compounds within DEP are the drivers of biological responses (Hirano *et al.*, 2003; Siegel *et al.*, 2004).

DEP-OE was taken forward to study the signalling pathways involved in the activation of airway sensory nerves, with the next aim being to determine the potential role for TRP channels. DEP has been linked to the activation of TRPA1, TRPV1 and TRPV4 (Teles *et al.*, 2009; Deering-Rice *et al.*, 2011; Li *et al.*, 2011), and these same TRP channels can activate airway sensory nerves (Birrell *et al.*, 2009; Nasra & Belvisi, 2009; Grace *et al.*, 2012; Bonvini *et al.*, 2016). The expression of all these channels was confirmed using RT-PCR in whole ganglia. Using single cell RT-PCR in airway specific neurons, only TRPV1 and TRPA1 mRNA were widely detected however the functional effects of TRPV4 agonists in airway neurons have previously been confirmed (Bonvini *et al.*, 2016). Next, the effects of selective antagonists for these TRP channels were investigated. In the isolated guinea pig vagus nerve, the TRPA1 antagonist Jan 130 (10μM) significantly inhibited depolarisation induced by DEP-OE, whilst the TRPV1 antagonist Xention (100nM) and the TRPV4 antagonist GSK219 (10μM) had no effect. Jan 130 (10μM) also inhibited responses to DEP-OE in human vagal tissue. To

compliment these pharmacological results, the depolarisation induced by DEP-OE (1µg/ml) was investigated in $TRP^{-/-}$ mice. Responses to DEP-OE (1µg/ml) were significantly reduced in $TRPA1^{-/-}$ mice compared to WT controls, while responses in $TRPV1^{-/-}$ and $TRPV4^{-/-}$ mice were unaltered. The effect of Jan 130 was then investigated *in vivo*, where it was confirmed to inhibit action potential firing in response to aerosolised DEP-OE. Together these results demonstrated that the activation of the vagus nerve by DEP-OE is mediated by the TRPA1 channel in both animal models and that this mechanism was also applicable for humans. TRPA1 has been previously shown to be activated by air pollutants (Shapiro *et al.*, 2013), and Deering-Rice *et al.* (2011) also reported that activation of neurons by organic extracts of DEP was mediated by TRPA1. The activation of airway sensory nerves by TRPA1 has been shown to induce reflex responses such as cough and has also been implicated in asthmatic responses (Birrell *et al.*, 2009; Grace & Belvisi, 2011; Raemdonck *et al.*, 2012). Activation of TRPA1 therefore is a plausible mechanism by which DEP may be inducing airway reflexes that result in adverse respiratory symptoms.

Attention was then turned to the underlying mechanisms by which DEP can activate TRPA1. One of the main mechanisms by which TRPA1 can be activated is through the covalent modification of cysteine residues located on its N-terminus region (Takahashi *et al.*, 2008; Sadofsky *et al.*, 2011). This common mechanism enables a diverse range of structurally distinct compounds to be able to activate TRPA1 as they possess electrophilic properties (Macpherson *et al.*, 2007). TRPA1 agonists include numerous products of oxidant stress and therefore TRPA1 is also known as an oxidative stress sensor (Kim & Hwang, 2013). Oxidative stress is also one of the key mechanisms by which air pollutants are thought to exert physiological effects (Kelly, 2003; Ghio *et al.*, 2012a).

To determine whether oxidative stress was implicated in the activation of TRPA1 by DEP, firstly a concentration response to H_2O_2, a classic oxidant stressor, was carried out and demonstrated that oxidant stress activated the isolated vagus nerve. Using Jan 130 (10µM), it was shown that H_2O_2 induced depolarisation was mediated by TRPA1, as previously reported (Sawada *et al.*, 2008b). The effect of antioxidants was then investigated, and NAC (1mM) and GSH (400µM) inhibited responses to H_2O_2 (10mM) and acrolein (300µM) via neutralisation of electrophilic regions, but had no effect on TRPV1 (capsaicin 1µM) induced responses. These results validated the use of antioxidants as a tool to probe oxidative stress as mechanism. NAC has been shown to inhibit responses in humans in controlled DEP exposure experiments (Carlsten *et al.*, 2014), therefore NAC was selected as the antioxidant to test against DEP-OE. NAC (1mM) inhibited DEP-OE (1µg/ml) induced depolarisation in the isolated guinea pig vagus nerve and this key result was also paralleled in human tissue.

Together these results indicate that neuronal responses to DEP-OE are driven by the generation of oxidative stress.

The next aim was to determine which compounds in DEP-OE were responsible for the observed activation of the vagus nerve. A vast array of potential compounds can be identified from the manufacturer's certificate of analysis for SRM-1975. Phenanthrene was found to be present in a relatively high concentration compared to other compounds in SRM-1975 and is also one of the 16 priority PAHs identified by the EPA to be monitored in the environment. For these reasons, the effect of phenanthrene was investigated. Phenanthrene was shown to activate the vagus nerve in a concentration dependent manner and this effect was inhibited by Jan 130, suggesting that phenanthrene may be one of the key components of DEP-OE that activates TRPA1. Traditionally, PAHs are through to exert their toxic effects through activation of the aryl hydrocarbon receptor (AhR), a ligand gated transcription factor that mediates the upregulation of a wide range of toxin response genes (Hahn, 2002; Billiard, 2006). The cellular responses mediated by AhR should occur after hours, rather than the more immediate responses neuronal responses that were observed (Rowlands & Gustafsson, 1997). Nevertheless, the effects of two structurally distinct AhR antagonists were investigated. Both CH-223191 (10 μM) and TMF (10 μM) inhibited response to both phenanthrene (10^{-9} M) and DEP-OE (1μg/ml). AhR antagonism had no effect on the TRPV1 (capsaicin) and TRPA1 (acrolein) induced responses, highlighting that its effects were specific to PAHs and not for TRP agonists that are classical activators of the vagus nerve. Preliminary investigations indicated that responses to DEP-OE were also reduced in $AhR^{-/-}$ mice compared to WT controls, while TRP agonist responses were unaltered. Together, these results highlight a novel role for AhR in mediating immediate responses in airway sensory nerves in response to environmental pollutants.

Interestingly, the ability for AhR to generate oxidative stress has already been well established in the literature to be mediated by the increased expression of downstream detoxification enzymes such as CYP1A1. However as previously mentioned, upregulation of target genes is unlikely to be the mechanism underlying neuronal activation. AhR has been found to be associated with the mitochondria, which is a major source of ROS within the cell and mitochondrial ROS has been shown to activate sensory nerves(Senft *et al.*, 2002; Hadley *et al.*, 2012; Nesuashvili *et al.*, 2013a). Antimycin A, an inducer of mitochondrial ROS, activated the isolated vagus nerve in a concentration dependent manner. Antimycin A (20μM) induced depolarisation was blocked by MitoTEMPO (2μM), a mitochondrially targeted superoxide scavenger. MitoTEMPO had no effect on TRPA1 (acrolein) induced responses, demonstrating a specificity for mitochondrially induced ROS. MitoTEMPO inhibited responses to DEP-OE,

demonstrating the role of mitochondria ROS generation following DEP-OE exposure in airway sensory nerves.

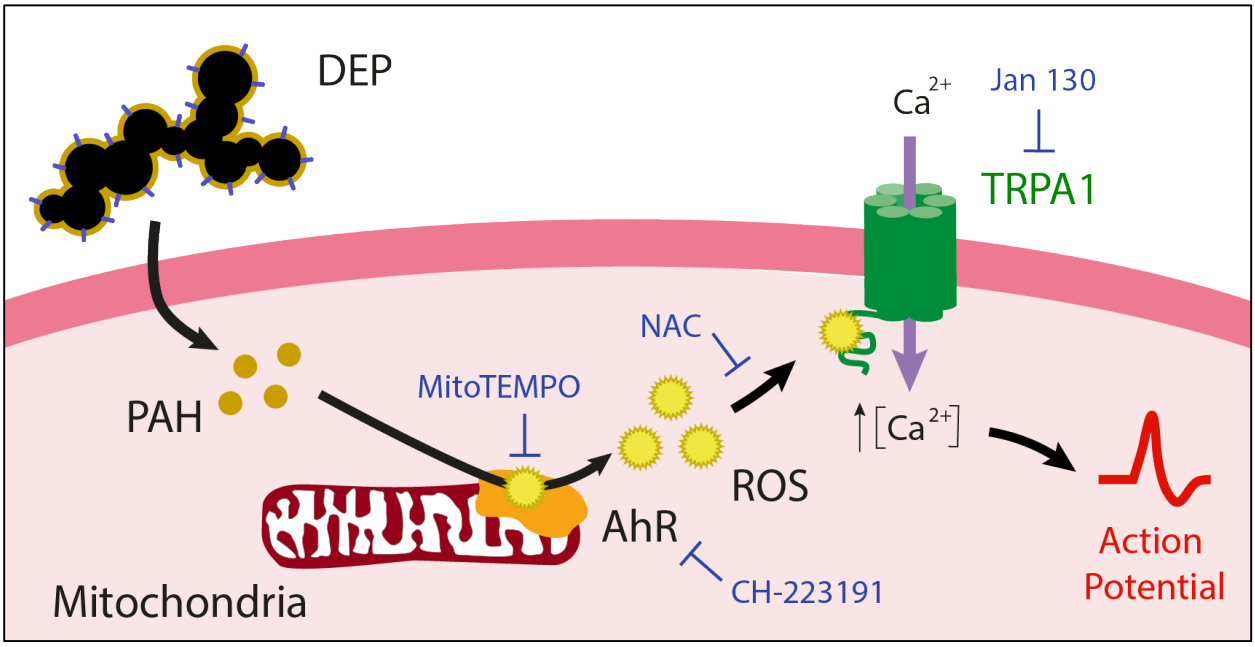


Figure 7.1: Schematic diagram illustrating the mechanisms by which DEP activate airway sensory nerves.

DEP inhaled into the lung contain PAHs embedded on their surfaces. PAHs act upon AhR associated with the mitochondrial to general mitochondrial ROS. Mitochondrial ROS activates TRPA1, resulting in an influx of Ca^{2+} into the cell and initiating action potential firing of the vagus nerve that leads to respiratory reflexes.

To conclude, the data within this thesis has identified a novel mechanism by which DEP particles can activate airway sensory nerves. In C-fibre afferents, PAHs present in DEP interact with AhR associated with neuronal mitochondria, generating mitochondrial ROS which subsequently activates TRPA1 (Fig 7.1). This pathway ultimate results in action potential firing of the nerve, which has been shown to result in a range of respiratory symptoms (Birrell *et al.*, 2009; Bonvini *et al.*, 2015). The wider implications of this knowledge may be that potential therapeutic treatments could be developed to protect individuals from the effects of air pollutants such as DEP. Potential targets could include TRPA1, AhR or antioxidants to combat mitochondrial oxidative stress. TRPA1 antagonists are currently undergoing preclinical trials, with drugs being developed both for pain relief and respiratory disorders (Andrade *et al.*, 2012; Preti *et al.*, 2012). MitoQ, another mitochondrial specific antioxidant displayed no adverse health effects noted during a 1 year trial in humans (Smith & Murphy, 2010), may therefore be more attractive targets to protect against the adverse effects of DEP. Oral administration of

mitochondrial antioxidant drugs allow for systemic bioavailability and protection from oxidative stress. Similarly, AhR antagonists may also be beneficial. However many of the existing AhR antagonists are not suitable as clinical drugs as they often exhibit partial agonist properties (Pernomian & da Silva, 2015). As CH-223191 and TMF appear to be more selective and lack agonist activity, further research into derivatives of these compounds to create pharmacologically suitable drugs is underway (Murray *et al.*, 2010; Zhao *et al.*, 2010; Choi *et al.*, 2012). Given that AhR is expressed throughout the body and can influence the expression of a wide range of genes including those linked to cancer, topical administration through inhaled aerosols may be preferred to prevent unwanted side effects.

However, 'prevention is better than cure'. For some individuals, it may be helpful therapeutic intervention based on the results of this thesis may be developed to help individuals that are severely and acutely affected by DEP. But ultimately there may be greater benefits for wider society to use the findings of this thesis to aid in the reduction of diesel emissions. The novel mechanisms identified in this thesis provide additional evidence for the ways in which DEP can be harmful, strengthening the case for further reductions in diesel emissions in urban environments. Governmental policy often relies upon scientific evidence. Equally, dissemination of this knowledge to the public can also influence decision makers to prioritise the dangers of air pollutants, particularly given the increased public awareness of urban air pollution in the wake of the recent Volkswagen emissions scandal. Further investigation is necessary to determine how neuronal mechanisms play a role in wider air pollution that exist beyond those what have been investigated in this thesis.

7.2 Limitations of this thesis

It is important to recognise that several limitations exist concerning the concerning the work carried out in this thesis. One such limitation concerned the use of SRM-2975 as the DEP selected for the basis of this thesis. While this SRM-2975 has been studied in the literature, its relevance to the health effects associated with roadside traffic emissions may be questioned as it originates from a forklift truck and has been shown to be relatively 'clean' DEP (Ball *et al.*, 2000; Jensen, 2006; McQueen *et al.*, 2007; Chaudhuri *et al.*, 2010; Masala *et al.*, 2011).

In terms of physicochemical characterisation, as outlined previously DLS was not an appropriate technique to measure particles that are heterogeneous in size. For this reason, cryo-EM was used to analyse the size and morphology of DEP. However, cyro-EM imaging was still carried out in liquids, as DEP was dispersed in Krebs solution. While these were

relevant for the *in vitro* studies carried out in these studies, in the urban environment DEP are suspended in the ambient air. DLS, despite its inappropriateness, still observed rapid aggregations of DEP in Krebs solution and particle aggregation rates are likely to be different in liquid or gaseous media. Another limitation is that the PAHs present in SRM-1975 were only characterised by the manufacturer, rather than being confirmed within the laboratory. The DCM solvent found in SRM-1975 was substituted for DMSO to facilitate the use with the laboratory equipment utilised within this thesis. It is possible that the resulting DEP-OE produced contained different levels of PAHs following this process. Analytical techniques such as gas chromatography/mass spectrometry could be utilised to quantify the levels of PAHs present in DEP-OE that have been used within this thesis.

Numerous experiments have been carried out using the *in vitro* isolated vagus nerve preparation. However, the isolated pieces of vagus nerve contains many other nerves in addition to airway afferents as the vagus nerve also innervates a range of organs throughout the body. Therefore, depolarisations measured using the isolated vagus nerve preparation may not necessarily be recorded from airway sensory nerves. Additionally, *in vivo* the termini of airway afferents are the regions of the nerves that will be exposed to DEP and it is unclear whether the same cellular components are present at the nerve trunk compared to the termini. In an effort to mitigate the issues with the *in vitro* isolated vagus nerve preparation, *in vivo* recordings of action potential firing was recorded in airway afferents. While benefiting from actively investigating responses mediated by termini of airway afferents, this method is technically challenging to carry out, relatively slow in throughput and costly to perform a complete study as only one experiment can be carried out per animal.

The majority of experiments also were carried out utilising guinea pig vagal tissue which may express different signalling proteins than those in human tissues. To overcome this limitation, key experiments were repeated using human vagal tissue to confirm that results from animal models was translatable. Unfortunately, translatable data can only be generated for the isolated vagus nerve technique as *in vivo* recordings are not possible due to its highly invasive nature.

7.3 Future studies

7.3.1 Investigate the effects of antioxidants and AhR antagonists on DEP-OE induced action potential firing *in vivo.*

In Chapter 6, it was demonstrated that the application of the general antioxidant NAC and the mitochondrial specific superoxide scavenger MitoTEMPO inhibited responses to DEP-OE in the *in vitro* isolated vagus nerve preparation. The specific AhR antagonists CH-223191 and TMF also inhibited responses to DEP-OE (1µg/ml) *in vitro*. However, due to time constraints it was not possible to test the effects of these compounds *in vivo* using airway single fibre recordings. Two repeated responses to DEP-OE (1µg/ml) would be obtained and then the animal treated with either NAC, MitoTEMPO, CH-23191, TMF or the relevant vehicle control and then the animal would be challenged again with DEP-OE (1µg/ml). Action potential firing would be recorded during each challenge.

7.3.2 Investigate the airway reflex responses induced by DEP-OE exposure.

This present study has identified the mechanisms by which DEP can activate airway sensory nerves *in vivo* and *in vitro*. However, these results have only demonstrated the activation of the vagus nerve, rather than demonstrate that subsequent respiratory symptoms can be initiated. Therefore, the physiological significance of DEP induced activation of airway sensory nerves still needs to be elucidated.

One of the most robust respiratory reflexes induced by airway sensory nerve activation is the cough reflex. Cough challenges can be carried out in both animals and man. For guinea pigs, animals would be placed into sealed chambers and exposed to aerosolized DEP. Trained observers would then count the number of coughs over five minutes of DEP challenge and five minutes following the challenge to ensure that any post-challenge cough was measured. Unfortunately, we did not have access to a diesel generator that could be connected to the animal cough chambers for such experiments. For human studies, collaborations could be formed with groups that routinely carry out such experiments. Subjects would be placed into chambers and exposed to either clean air or high concentrations of DEP (300µg/m^3) for two hours (Carlsten *et al.*, 2016) Subjects could also be equipped with personal cough monitoring devices that can be used to obtain objective measurements of cough counts during exposure (Marsden *et al.*, 2016).

7.3.3 Fluorescence of neurons exposed to DEP

To complement results obtained from *in vivo* studies recording action potentials from airway sensory nerves, *in vitro* calcium imaging of airway specific neurons could be carried out. This

would confirm that DEP directly activate airway innervating neurons without the additional presence of the complex *in vivo* lung environment. Guinea pigs would be intranasally administered with DiI, a retrograde tracer dye and 14 days later vagal ganglia neurons could be harvested. Following enzymatic digestion of the ganglia, the isolated neurons would be incubated with Fura-2AM, a calcium sensitive dye. Utilising light microscopy, neurons could firstly be identified as innervating the airway by the presence of the DiI (Fig 7.2A), then measurements of Ca^{2+} levels within the cell could be recorded in response to challenge with DEP (Fig 7.2B). Ca^{2+} influx is an important step following the activation of TRP channels and is a marker of neuronal activation.

Initial experiments have already been carried out (as described in Chapter 2). The effect of DEP and other oxidative stimuli were investigated in airway jugular and nodose ganglia was investigated according to the following protocol. Firstly, a calcium response to hyperpotassium (K_{50}) solution was obtained by which subsequent responses would be normalised to. Next, a vehicle control was applied for 4 mins, then the cell was washed for 5 mins. The cell was then challenged with either DEP-OE (1µg/ml or 10 µg/ml), H_2O_2 (10mM) or antimycin A (20µM) for 4 mins, before washing for 5 mins or until the calcium signal returned to baseline. Finally, neurons were challenged with acrolein (30µM) as a positive control for TRPA1 responses. From the initial results obtain, DEP-OE (1µg/ml) does not induce calcium flux in either jugular or nodose neurons. However, both H_2O_2 (10mM) and antimycin A (20µM) both appear to induce calcium flux in jugular neurons, while only H_2O_2 activated nodose neurons (Fig 7.2C/D/E/F). It should be noted that not all neurons responded to these stimuli, however equally only a subset of neurons responded to acrolein (30µM) as a TRPA1 positive control. Further experiments are necessary, to determine the reason for the lack of response to DEP-OE in isolated neurons as generally results from calcium imaging experiments are expected to mirror those obtained from the isolated vagus nerve preparation. Potential reasons for this discrepancy may be augmented protection to oxidative stress in the cell bodies of neurons compared to vagal afferent fibres or an indirect effect of oxidant stress on a non neuronal cell.

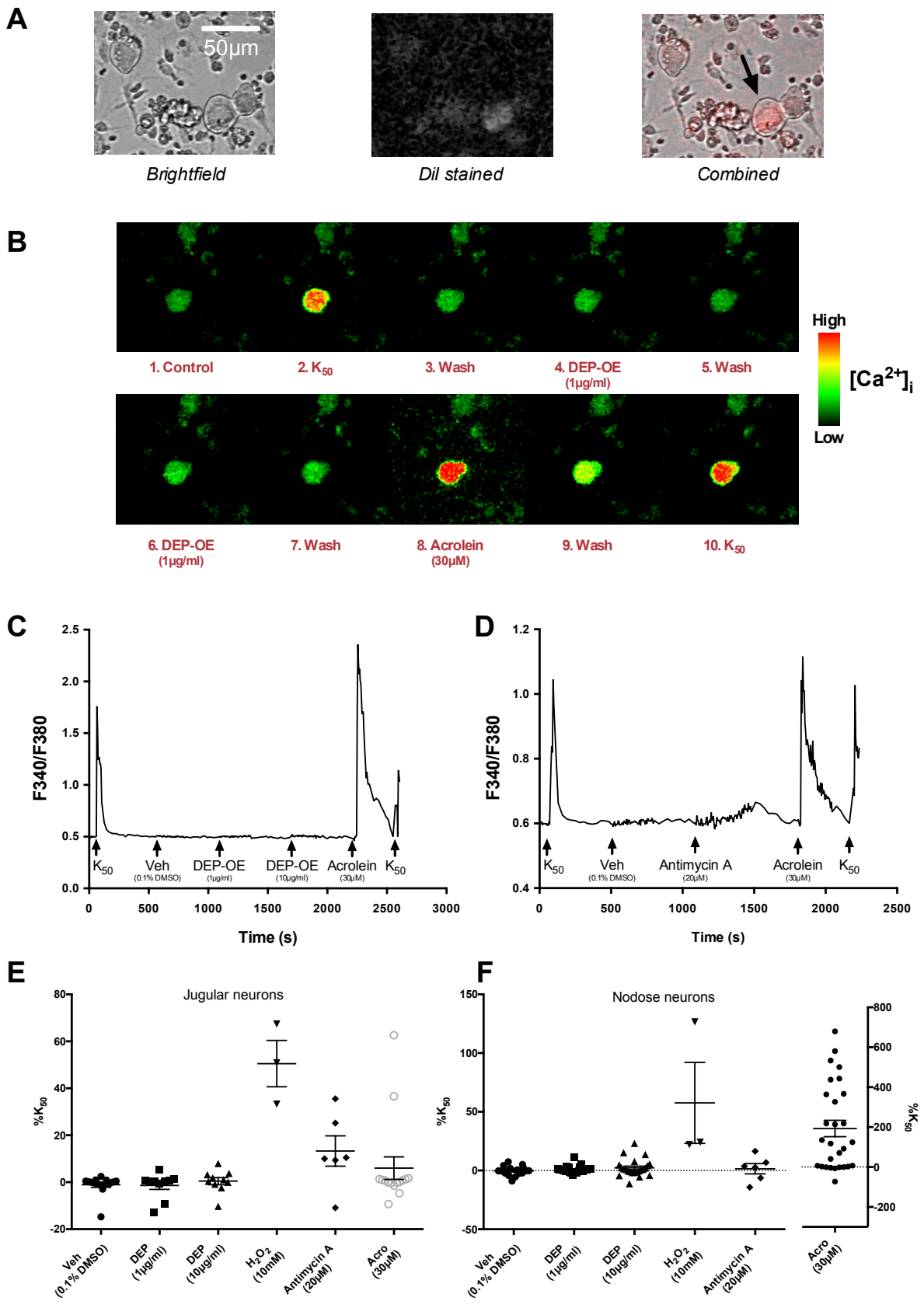


Figure 7.2: Effect of DEP and other agonists on calcium signal in airway neurons.

A) *Neurons that innervated the airways (highlighted by black arrow) were identified by the presence of DiI staining.* ***B)*** *Images of the calcium signal generated by the calcium sensitive dye Fura-2AM in*

response to agonists. Red colour signifies increased calcium signal. ***C)*** *Representative trace of nodose neuron exposed to K_{50}, vehicle (0.1% DMSO), DEP (1µg/ml and 10µg/ml) and acrolein (30µM). The neuron was challenged with a final K_{50} stimulation to confirm viability.* ***D)*** *Representative trace of nodose neuron exposed to K_{50}, vehicle (0.1% DMSO), antimycin A (20µM) and acrolein (30µM). The neuron was challenged with a final K_{50} stimulation to confirm viability.* ***E)*** *Mean data of normalised calcium responses to various agonists in airway specific jugular neurons.* ***F)*** *Mean data of normalised calcium responses to various agonists in airway specific jugular neurons. (N=4) Data represented as mean ± SEM.*

Fluorescence imaging would also allow for the levels of oxidant stress within neurons to be recorded following exposure to DEP or other stimuli. As with calcium imaging, neurons would firstly be enzymatically digested and cultured overnight. Prior to imaging, the cells would be incubated with oxidant stress sensitive dye dihydroethidium (DHE – 5µM) for 30 mins before being thoroughly washed with PBS. DHE exhibits blue-fluorescence until it is oxidised, and thus by exciting the cells at 480 nm and recording at 510 nm, therefore imaging using a fluorescent microscope would allow for measurements to be made of the levels of oxidative stress within neurons. The application antioxidants or antagonists for the upstream AhR may inhibit oxidative stress generated in response to DEP as measured by this technique, further validating the mechanistic pathway identified in this thesis.

7.3.4 Profiling the effects and properties of other DEP and PM.

One of the key objectives of future work will be to profile neuronal responses to different types of DEP and PM. The mechanisms identified in this present study may be relevant over a range of pollutants, and preliminary investigations into this have already been carried out.

Human studies utilising an alternative form of DEP generated by a commercial diesel generator engine found that increased airway responsiveness after 2 hours of DEP (300µg/m^3) exposure could be prevented with pretreatment with NAC (Carlsten *et al.*, 2014). These human exposure studies therefore have mimicked ‘real world’ exposures to DEP that may occur in an occupational setting and demonstrated that this resulted in respiratory symptoms. Samples of the same DEP were captured on Teflon particulate filters (Fig 7.3A) and extracted via ethanol based extraction. This alternative DEP (1µg/ml) was found to depolarise the vagus nerve in a similar manner to SRM-2975 that was the focus of this thesis. Additionally, the application of Jan 130 (10µM) to inhibit TRPA1 or NAC (1mM) as an antioxidant significantly inhibited responses to this alternative DEP (Fig 7.3B), potentially

indicating that the neuronal mechanisms identified in this study are applicable to DEP from a variety of sources.

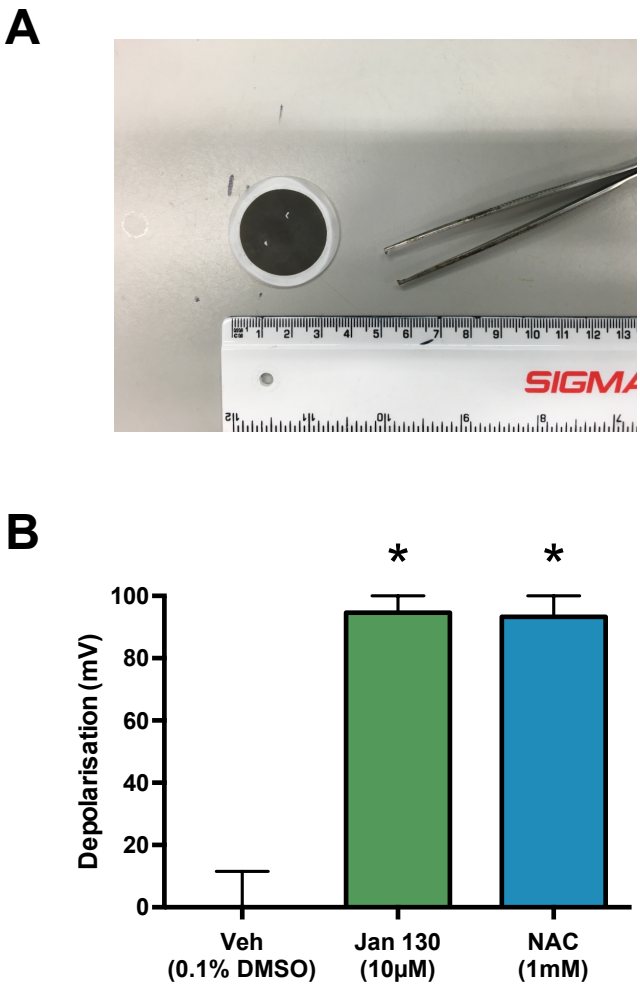


Figure 7.3: Effect of TRPA1 antagonism and antioxidant application in an alternative form of DEP.

A) *DEP generated from a commercial diesel generator engine was collected on particulate filters and then removed using an ethanol based extraction* ***B)*** *Depolarisation induced by the alternative form of DEP (1μg/ml) was significantly inhibited by the application of the TRPA1 antagonist (10μM) or the antioxidant NAC (1mM), while treatment with vehicle (0.1% DMSO) had no effect. (n=3-5). Data is expressed as mean ± SEM of the percentage inhibition as calculated by comparing DEP responses in the presence and absence of antagonist within the same nerve. * denotes statistical significance (P < 0.05), calculated using a paired t-test*

Other forms of DEP can also be obtained and further work is necessary to characterise how differences in physicochemical composition affect the activation of the vagus nerve. Several studies have highlighted that SRM-2975 is a relatively 'clean' DEP in terms of organic hydrocarbon content and embedded transition metals. It may be possible that other forms of

DEP with different characteristics will be able activate the vagus nerve in a more potent manner. In addition to fork-lift derived truck DEP, NIST also supply SRM-1650b which is has been collected from several direct injection four-cycle diesel engines. DEP could also be collected from sampling stations based near heavily trafficked roads to obtain samples more relevant to real world exposures. A combination of *in vitro* and *in vivo* techniques could be utilised to compare the physiological effects of different DEP, although the isolated vagus nerve technique would likely be preferred as an initial approach due to speed.

Any physiological investigations using other forms of DEP would have to be carried out using simultaneous physiochemical characterisation. Techniques such as gas chromatography/mass spectrometry can be used to identify and quantify the organic compounds present in DEP. Such characterisation studies would enable associations to be made between the concentrations of PAHs in various DEP and their respective physiological effects. It may then be possible for predictions to be made with regards to the potential of DEP to activate airway sensory nerves. Informed decisions could then be made as to the types compounds present in DEP that are most harmful, and potentially influence changes in diesel powered motor vehicle technology or governmental policies aiming to reduce urban diesel emissions.

7.3.5 Determining the effects of DEP exposure in disease models

The experiments carried out in this thesis utilised tissue from healthy animals, however an enhanced sensitivity to the effects of DEP are observed in individuals with underlying respiratory diseases. It will be important to determine whether animal models of disease also possess enhanced sensitivity to the effects of DEP and whether this has a neuronal basis.

Several studies have highlighted that associations between DEP exposure and respiratory symptoms are more commonly found in asthmatic individuals. To probe this link mechanistically, rodent models of asthma that have been previously developed in our laboratory could be combined with repeated DEP exposure (Raemdonck *et al.*, 2012). Brown Norway rats would be sensitised on day 0, day 14 and day 21 with OVA (100 µg/rat) with Alum (20 mg/rat aluminium hydroxide – 20 mg/rat and magnesium hydroxide – 20 mg/rat). During this time period, rats would be exposed every day to either air or to diesel exhaust generated by a diesel engine. By characterising the levels of DEP emitted from the diesel engine, a dilution system could be constructed to ensure that the concentrations of DEP are controlled. On day 28, rats would be challenged with either aerosolised saline or OVA (1% w/v) and PenH

could be used as a marker of increased airway responsiveness to confirm the asthma phenotype. It would be interesting to determine whether the additional burden of continuous DEP exposure increases the severity of the asthmatic response following OVA challenge.

Using such a model, the vagus nerve and ganglia could also be harvested to profile whether phenotypic changes are preserved *in vitro* by challenging with agonists such as capsaicin or acrolein and determining whether responses were augmented in DEP treated animals. Additionally, NAC and CH-223191 could be administered during the sensitisation period to determine whether the mechanisms highlighted in this thesis are critical to any potential sensitisation. These experiments would provide evidence for a neuronal basis for the increased susceptibility to the effects of DEP in respiratory diseases.

COPD is a disease that is thought to be closely associated with smoking, and patients with COPD also possess increased sensitivity to DEP exposure (Bayram *et al.*, 2009; Hart *et al.*, 2012; Sinharay *et al.*, 2013). Over eight days, guinea pigs would be exposed to cigarette smoke in for one hour twice a day to develop a COPD-like phenotype with airway inflammation. Guinea pigs treated in this manner display increased cough sensitivity *in vivo* and increased responses to capsaicin *in vitro* in a similar manner to COPD patients (Belvisi *et al.*, 2016). Rats are another animal species that respond in a similar manner (unpublished data from our laboratory). Preliminary investigations of the effects of DEP in a rat model sensitised with cigarette smoke have already been carried out. Rats were sensitised with cigarettes as outlined above. The animals were sacrificed with an overdose of pentobarbital and the vagus nerves removed. A concentration response to DEP was carried out using the isolated vagus nerve technique, and preliminary results may indicate that rats exposed to cigarette smoke display a slight increase in sensitivity to DEP (Fig 7.4). The increased sensitivity to TRPA1 stimuli has not been previously published, unlike increased TRPV1 sensitivity, although these results have been observed within our laboratory. It should be noted that cigarette smoke treatment does not appear to indiscriminately increase neuronal sensitivity to agonists, as responses to PGE_2 in such models are in fact decreased (Belvisi *et al.*, 2016).

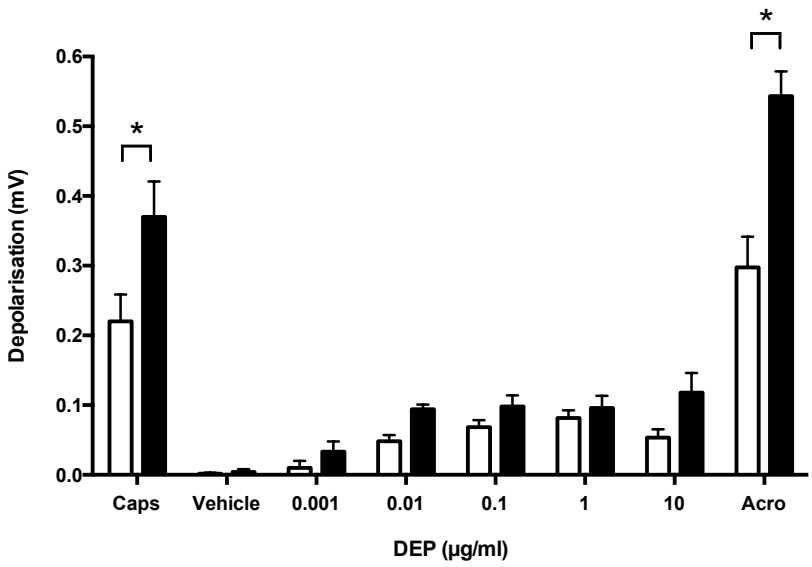


Figure 7.4: Concentration response to DEP in Brown Norway rats treated with either air or cigarette smoke.

*Brown Norway rats were exposed to either air (n=4-6) or cigarette smoke (n=3-5) for one hour twice a day for 8 days. After this period, the vagus nerve was harvested and a concentration response was carried out to DEP (0.001 to 10µg/ml). The vagus nerve was also challenged with capsaicin (1µM) and acrolein (300µM) and responses to these agonists were also increased in smoke treated rats. Data is expressed as mean ± SEM. * denotes statistical significance (P < 0.05), calculated using 2 way ANOVA with a post-hoc Sidak's multiple comparisons test comparing air with smoke treated groups.*

7.3.6 Development of a diesel exposure animal model

Repeated exposure to cigarette smoke, another environmental pollutant, also alters neuronal phenotypes (Belvisi *et al.*, 2016). Therefore, it would be interesting to determine whether repeated exposure to DEP can influence neuronal phenotypes in a similar manner. Using a diesel engine attached to sealed chambers, animals could be exposed to diesel emissions to mimic the exposures encountered in urban environments. By characterising the levels of DEP emitted from the diesel engine, a dilution system could be constructed to ensure that the concentrations of DEP are controlled. Following DEP exposure, animals could be utilised for *in vivo* cough challenges or airway single fibre recordings in response to tussive stimuli. Additionally, vagal nerve tissue and ganglia neurons could also be harvested to determine whether sensitivity to tussive stimuli were also altered *in vitro*. To determine an appropriate concentration and times for DEP exposure, either existing studies could be emulated or more in depth experiments could be carried out involving concentration response and time courses. As previous studies have not specifically addressed the role of airway sensory nerves, it may be more appropriate to take the latter approach.

7.4 Concluding remarks

From the results of this thesis, it has been demonstrated that DEP are capable of activating airway sensory nerves. These findings provide mechanistic explanations for the respiratory effects of DEP exposure observed in epidemiological studies. Additionally, cellular mechanisms have been identified that highlight the importance of the organic components of DEP as well as role of TRPA1 and mitochondrial oxidative stress in the activation of airway sensory nerves. This thesis has also demonstrated a novel role for AhR in mediating cellular signalling events that do not involve transcription and translation.

Ultimately, the findings of this thesis and the subsequent work that follows will provide additional evidence to drive a change in the levels of diesel emissions that individuals are exposed to in an urban environment. Treatments could also be developed to inhibit the activation of airway sensory nerves upon exposure to DEP, especially in susceptible population groups. However, efforts should be made to reduce the levels of DEP in the urban environment. A combination of influencing change in government policy and public opinion will hopefully drive reductions in diesel emissions, either by improvements to emission reduction technology or a complete shift away from fossil fuel powered motor vehicles. Whatever the method by which diesel emissions are reduced, it is clear from epidemiological studies that such changes will provide numerous benefits to public health and should be an objective that society strives for.

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Appendix:

The table below describes the substances used throughout this thesis and their original sources. Vehicles/Diluents have been added where applicable.

Substance	Source	Vehicle/Diluent (where applicable)
6,2',4'-Trimethoxyflavone (TMF)	Tocris	0.1% DMSO in Krebs
Acrolein	Sigma-Aldrich	0.1% DMSO in Krebs
Antimycin A	Sigma-Aldrich	0.1% DMSO in Krebs
$CaCl_2$	VWR	-
Capsaicin	Sigma-Aldrich	0.1% DMSO in Krebs
CH-223191	Sigma-Aldrich	0.1% DMSO in Krebs
Citric Acid	Sigma-Aldrich	0.9% saline
Dichloromethane (DCM)	Sigma-Aldrich	-
DiI (DiIC18(3), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)	Invitrogen	2% ethanol in 0.9% saline
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	-
Ethanol	VWR	-
F12	Invitrogen	-
Fluo–2-AM	Invitrogen	Extracellular Solution
Glucose	Sigma-Aldrich	-
GSK1016790a	Sigma-Aldrich	0.1% DMSO in Krebs
GSK2193874	Almirall	0.1% DMSO in Krebs
HEPES	Sigma-Aldrich	-
Hydrogen Peroxide (H_2O_2)	VWR	Krebs
Janssen 130	Almirall	0.1% DMSO in Krebs (in vitro). 1% methyl cellulose in saline (in vivo)
KH_2PO_4	VWR	-
Methyl Cellulose	Sigma-Aldrich	-
$MgSO_4$	VWR	-
MitoTEMPO	Sigma-Aldrich	0.1% DMSO in Krebs
N-Acetyl-L-cysteine (NAC)	Sigma-Aldrich	Krebs

NaCl	VWR	-
NaH_2PO_4	VWR	-
$NaHCO_3$	VWR	-
Nuclease free water	Promega	-
PBS	Sigma-Aldrich	-
Petroleum Jelly	Vaseline	-
Phenanthrene	Gift from Dr. Ian Mudway (King's College)	0.1% DMSO in Krebs
Reduced Glutathione (GSH)	Sigma-Aldrich	Krebs
Safeview	NBS Biologicals Ltd	-
SRM-1975	NIST	0.1% DMSO in Krebs (after solvent replacement with DMSO)
SRM-2975	NIST	Krebs
Tween80	Sigma-Aldrich	-
Xention D0501	Ario Pharma Ltd	0.1% DMSO in Krebs