

# **INTERACTION OF ANAESTHETICS WITH THE CAROTID BODY GLOMUS CELL AND THE BACKGROUND POTASSIUM CHANNEL RESPONSE TO HYPOXIA**

**A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**



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Dedicated to Rachel, Joseph and Annabel

## **ABSTRACT**

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### **INTERACTION OF ANAESTHETICS WITH THE CAROTID BODY GLOMUS CELL AND THE BACKGROUND POTASSIUM CHANNEL RESPONSE TO HYPOXIA**

This thesis investigates the role anaesthetics play in the depression of the acute hypoxic and hypercapnic ventilatory response. Particular attention is paid to TASK (TWIK related acid sensitive potassium) channels that are known to be sensitive to both hypoxia and the volatile anaesthetics.

After chapters relating to a general introduction and methods, chapter 3 describes studies into the hypoxic and hypercapnic responses of mice with genetic ablation of TASK 1 and/or TASK 3. By using whole animal unrestrained plethysmography, it was demonstrated that any loss of TASK channels resulted in a depression of ventilatory responses to hypoxia and hypercapnia. In isocapnic hypoxia, there is a genotype specific depression by anaesthetics of the remaining AHVR, depending on whether halothane or isoflurane is inspired.

Chapter 4 establishes that propofol depresses the chemosensory responses of carotid body glomus cells. Using ratiometric fluorimetry to measure the intracellular calcium concentration in isolated neonatal rat glomus cells, it was demonstrated that propofol depresses the normal cellular response to both hypoxia and hypercapnia. Using pharmacological modulators of GABA and nAChR, and single channel patch clamp electrophysiology of TASK, it is apparent that this depression is independent of these candidate mechanisms.

Chapter 5 describes the effects of the anaesthetic dexmedetomidine at two levels of the hypoxic chemoreflex arc in rodents. Neither isolated rat glomus cells, nor unrestrained mice show significant depression of hypoxic responses when dexmedetomidine is present.

Chapter 6 illustrates the potential role for specific antagonism of TASK channels in stimulating the carotid body. Two TASK channel blockers, A1899 (TASK 1 antagonist) and PK-THPP (TASK 3 antagonist) are shown to cause a significant influx of calcium into isolated glomus cells, and an augmentation in the glomus cell response to hypoxia, which persists in the presence of isoflurane.

Overall, this thesis provides evidence that both TASK 1 and TASK 3 channels are essential for normal hypoxic chemosensitivity. It has extended the knowledge that anaesthetics depress glomus cell chemosensation, by demonstrating that propofol too causes this effect. It also proposes that dexmedetomidine may have greater utility in sparing of ventilatory reflexes. By selectively blocking TASK channels, glomus cells can be stimulated, and exploiting this mechanism would be useful in minimising anaesthetic-induced depression of ventilation.

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**Chapter 1:**

**INTRODUCTION**

## **Overview**

Hypoxia is the syndrome of inadequate regional or systemic oxygen delivery or utilisation, and can be observed in a variety of pathological states as well as in hypobaric environments such as at altitude. The human physiological response to tissue hypoxia is characterised by a multi-system reflex to restore adequate oxygenation. In order to achieve this in the short term, ventilation and cardiac output increase and with sustained hypoxia erythropoiesis affects an increase in the oxygen carrying capacity of the circulation. The acute hypoxic ventilatory response (AHVR) describes the second-to-minute temporal adjustment of breathing to a change in oxygen tension of the blood.

General anaesthetics, both inhalational (volatile) and intravenous, depress the human physiological response to hypoxia. The mechanisms underlying this phenomenon are not fully understood, nor are there specific pharmacological strategies available to reverse this depression.

The  $K_{2P}$  family of potassium channels generate a “leak” current that maintains the negative membrane potential of excitable cells. Among the many tissues in which  $K_{2P}$  channels are functional, cells of the carotid body have been demonstrated to avidly express the TASK (TWIK like acid sensitive potassium channel) species of this protein family. TASK channels are oxygen sensitive, manifest as an open-probability directly correlated with oxygen tension. In a hypoxic milieu, the decrease in TASK associated potassium leak current results in membrane depolarisation and activation of a host of voltage gated channels, calcium influx and neurotransmitter release to closely associated afferent nerve terminals. TASK opening is not only influenced by hypoxia but is also inhibited

by acidosis and augmented by the volatile anaesthetic halothane. Given the rapid polymodal chemosensitivity of TASK channels and their halothane sensitivity, they are a plausible candidate for mediating the observed anaesthetic depression of AHVR.

### **Clinical hypoxia**

During air-breathing, total body oxygen stores are primarily determined by the volume of the functional residual capacity, or alveolar reserve volume, of the lungs. Oxygen flux is high, with total body consumption at ~250 ml/minute at rest rising to >6000 ml/minute during physiological stress (Hagerman, 1984). With such a large demand range, but a relatively fixed reserve, tissue oxygen supply requires rapid changes in ventilation to maintain aerobic metabolism. Prolonged periods of hypoxaemia are known to have detrimental effects on the brain (Erecinska and Silver, 2001), myocardium (Kloner and Jennings, 2001), wound healing (Sen, 2009) and immune function (Palazon et al., 2012). On an individual level, there is likely a threshold time at a defined level of hypoxaemia that could be harmful in the acute setting. For traumatic brain injured patients for example this may be a very short period at mild hypoxia. Others are likely to be more resilient.

Acute hypoxaemia is detected by the carotid bodies, generating neural afferent signals to the central nervous system respiratory control mechanisms. The result of this reflex loop is an increase in minute ventilation, the AHVR. Any perturbation of this mechanism has the potential to cause harm.

The carotid body is also stimulated by acidaemia and hypercapnia, is sensitive to hypoglycaemia (Bin-Jaliah et al., 2004) and can be implicated in the

pathophysiology of hypertension (McBryde et al., 2013), making it a polymodal chemosensor.

### **Anaesthetic depression of ventilatory responses**

#### **Anaesthetic morbidity**

Approximately 2.9 million general anaesthetics are delivered each year in the United Kingdom (Sury et al., 2014). In current worldwide clinical practise over 80% of these cases anaesthesia will be maintained by an inhalational anaesthetic agent (Myles et al., 2007; Sury et al., 2014). In the majority of the remaining cases an intravenous infusion of propofol is used. Post-operative complications that render patients vulnerable to hypoxaemia are common, such as atelectasis (7.5%) (Myles et al., 2007) and airway obstruction (6.9%) (Hines et al., 1992). It is of no coincidence that prior to the advent of pulse oximetry to measure haemoglobin oxygen saturation in real time, hypoxaemia was the leading cause of anaesthetic mortality. Concurrent with the universal adoption of pulse oximetry in developed countries, anaesthetic related deaths have decreased 20-fold. However, hypoxia and airway problems still occur.

Both volatile anaesthetic agents and propofol depress AHVR at doses that persist well into the post-operative phase of anaesthesia (Knill and Gelb, 1978; Nagyova et al., 1995; Pandit, 2002; Lockwood, 2010).

Herein lies the clinical problem: a commonly encountered complication (hypoxia) coincides with the normally protective mechanisms being obtunded. As Knill and Gelb (1982) famously put it, “are the watchdogs sleeping?” referring to the peripheral chemoreflex being switched off by anaesthesia.

The mechanisms by which these diverse drugs have similar (and clinically detrimental) effects are not fully understood, but evidence suggests that both may be mediated by depression of the afferent limb of the peripheral chemoreflex loop (Jonsson et al., 2005; Pandit and Buckler, 2009; Pandit et al., 2010b).

### **Link between chemosensation and anaesthesia**

Though the carotid bodies have now unequivocally been shown to be adversely affected by a wide array of anaesthetic agents, this was not always the case. In fact, part of the fascination of the anaesthetic fraternity with the carotid body arose because of a perceived resilience (Rosenfeld, 1936) of hypoxic ventilatory responses in the presence of anaesthetics. This view persisted, especially in the case of animal experiments, until the detailed study of halothane in the 1960s and 1970s (Brandstater et al., 1965; Munson et al., 1966; Weiskopf et al., 1974) established the depression of the peripheral chemoreceptor was indeed significant in both animals and humans. It is clear that one reason for this vacillation was disentangling not only the peripheral from the central limb of the reflex, but also the interdependence of CO<sub>2</sub> and O<sub>2</sub> and their interaction with the carotid body. As clinical practice moved from halothane to the novel agents enflurane, isoflurane and sevoflurane, the debate continued: could these agents be superior with respect to their depression of ventilatory reflexes? To this end, comparative studies have shown consistently that the volatile anaesthetics depress AHVR across species (Temp et al., 1992; Nagyova et al., 1994; Karanovic et al., 2010) and with a characteristic order of potency of halothane > isoflurane > sevoflurane. That is not to say that they are not depressive at the central limb of the chemoreflex; they are, and the level of

arousal or pain influences study outcomes if not controlled for (Temp et al., 1992; Temp et al., 1994; Sarton et al., 1996; Pandit, 2002; Pandit and Moreau, 2005). Audiovisual (AV) stimulation (but not pain) reverses the depression of AHVR by sevoflurane, but neither pain nor AV stimulation influence halothane depression of AHVR. With such complex, multisystem effects of anaesthetic agents, the general molecular mechanisms by which hypnotic agents act must be considered.

### **General mechanisms of anaesthetic action and hypoxia sensing**

#### **K<sub>2P</sub> channels**

The K<sub>2P</sub> family of potassium channels are characterised by four transmembrane domains and two pore-forming domains. They contribute to resting membrane potential, and are weakly inwardly rectifying. To form a functional channel, two K<sub>2P</sub> subunits form a dimer, which can be both homomeric or heteromeric. As there are 15 isoforms, the classification is into six subfamilies distinct in structure and/or function. Of these six groups, some are modulated by lipids, mechanical stress (TREK), pH (TWIK, TASK and TALK), and in specific tissues, hypoxia (TASK) (Renigunta et al., 2015).

#### ***Hypoxia sensitivity***

One of the most fascinating properties of potassium channels is their sensitivity to hypoxia. Over a short period of time, it was demonstrated by a number of groups that an oxygen sensitive potassium current was present in glomus cells (Lopez-Barneo et al., 1988; Peers, 1990a; Stea and Nurse, 1991). However, the exact biophysical properties took some time to be disentangled, in part due to species differences between rat (where the current appeared to be activated by

Ca<sup>2+</sup> (Peers, 1990a)) and rabbit (where it displayed voltage sensitivity (Lopez-Barneo et al., 1988)). However, to initiate membrane depolarisation, this conductance would need to be active at resting membrane potential (estimates are generally -50mV to -80mV). A channel fitting this requirement was described in rat glomus cells (Buckler, 1997), and paved the way to establishing TASK as the prime candidate for initiating membrane depolarisation in response to hypoxia. Subsequent work has expanded this to include a role for mitochondria in the regulation of TASK 1 and TASK 3 response to hypoxia (Buckler and Turner, 2013; Turner and Buckler, 2013).

#### ***Anaesthetic sensitivity***

Prior to the formal identification of the K<sub>2P</sub> channel family, Franks and Lieb (1988) described a halothane activated potassium current in *Lymnaea stagnalis*, which was subsequently recognised to have the characteristics of TASK 1 (Andres-Enguix et al., 2007). This discovery was part of a paradigm shift in the 1980s, from the long held “lipid theory” of anaesthetic action, to a model that included volatile anaesthetics acting on proteins, in this case an ion channel. Mammalian TASK (and TREK) channels were also found to be activated by halothane and isoflurane (Patel et al., 1999), extending the importance of this channel family as a likely target for anaesthetics. When expressed in *Xenopus* oocytes, TASK 1 channels retained sensitivity to etomidate, but not propofol (Putzke et al., 2007), though native TASK channels have never been studied with respect to the latter.

Mouse knockouts of TASK channels generally show an altered susceptibility to the hypnotic effects of both the volatile and intravenous anaesthetics (Linden et

al., 2006; Linden et al., 2007), though neither the  $K_{2P}$  subfamilies nor anaesthetics classes have been comprehensively interrogated. Loss of righting reflex, a proxy for hypnotic effect of drugs, is less influenced by volatile agents in TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup> and TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup>, but there is inconsistency with agents: halothane has an less effect in TASK 1<sup>-/-</sup>, but a similar effect to controls in TASK 3<sup>-/-</sup> whereas the converse is true for isoflurane. In the same series of studies, Linden et al. (2006) noted that TASK 1<sup>-/-</sup> mice were less sensitive to the sedative effects of dexmedetomidine (an  $\alpha_2$ -receptor adrenoceptor agonist), potentially implicating TASK in the regulation of central adrenergic mechanisms.

#### ***Anaesthetic influence on hypoxia sensing***

The property of both oxygen and anaesthetic sensitivity of TASK channels converged with the identification of the background potassium current as having the properties of TASK 1. This was important, as it established halothane as a prototypical channel agonist, and posed TASK as a hypothetical target for the anaesthetic depression of hypoxic responses.

Native channels are often not homomeric and the heteromultimeric  $K_B$  (native TASK) channels are likely to be made up of TASK-1 and TASK-3 subunits (Kcnk3 and Kcnk9 genes respectively) (Turner and Buckler, 2013). The heteromultimer is thought to contribute the major part of the oxygen sensitive background potassium conductance of glomus cells, with homodimeric TASK-1 and -3 channels also functionally expressed in these cells (Kim et al., 2009; Turner and Buckler, 2013). However, the volatile anaesthetic agents differentially agonise the subtypes of  $K_B$  channels; halothane is indiscriminate while isoflurane has little effect on TASK-1 homomers (Berg et al., 2004). It is

this heterogeneity both in the expression and dimerization of channels as well as the seemingly drug/channel specific effects that makes this a complex mechanism to unravel.

However, the unique anaesthetic and oxygen sensitivity makes TASK channels a candidate for mediating the whole body effects of the anaesthetic-hypoxic interactions seen clinically and in animal studies. The specific order of potency for anaesthetic depression of AHVR is halothane > isoflurane > sevoflurane in these cells. The glomus cell  $[Ca^{2+}]_i$  response to hypoxia is depressed more by halothane than by isoflurane (Pandit et al., 2010b) or sevoflurane (Pandit and Buckler, 2009).  $K_B$  channel activity is influenced more by halothane than isoflurane (Pandit et al., 2010b) or sevoflurane (Huskens, Thesis 2015, unpublished). This specific order is conserved across species (Pandit, 2002; Karanovic et al., 2010) and through multiple levels of physiological organisation (Pandit, 2002; Pandit and Buckler, 2009; Karanovic et al., 2010; Pandit et al., 2010b).

### **GABA receptors**

The ionotropic GABA-A receptor is affected by all general anaesthetics except xenon (Franks, 2008). Even in the absence of the endogenous ligand, inhalational and intravenous anaesthetics can stabilise the channel in the open state, activating GABA-A (Rudolph and Antkowiak, 2004). Channel mutation analysis, both in vitro (Mihic et al., 1997) and in vivo (Jurd et al., 2003) have shown that anaesthetic action on GABA-A could be removed by small changes in the protein structure. This is of great significant as in vivo this would manifest as a decrease in anaesthetic potency for hypnosis.

## Chapter 1 - Introduction

GABA-A receptors are expressed throughout the nervous system, and though often studied at synapses, where the classic excitation-inhibition coupling allows rapid regulation of neuronal transmission, they are also found extra-synaptically where their affinity for both GABA and anaesthetics is much greater (Bai et al., 2001). This results in anaesthetics enhancing “tonic-inhibition” by low concentrations of GABA of these extra-synaptic receptors. This could explain how a diverse group of anaesthetics could disrupt neuronal network dynamics to effect hypnosis, despite widely differing  $EC_{50}$  values at the typical synaptic GABA-A receptor subtype (Franks, 2008).

To a lesser extent GABA-B, a metabotropic receptor, has shown some features of sensitivity to anaesthetics (Schwieler et al., 2003). Selective GABA-B agonism can cause sedation, though much of the work surrounding this as a pharmacological target has been related to the treatment of muscle spasticity and drug withdrawal syndromes.

There is good reason to suspect that GABA, the neurotransmitter, may be involved in hypoxic chemotransduction also. Midazolam, a benzodiazepine that enhances the activity of GABA receptors, depresses the AHVR in humans (Alexander and Gross, 1988). GABA subunits and mRNA are present in the carotid bodies of animals, and in these models benzodiazepines antagonise hypoxic chemosensitivity (Igarashi et al., 2009). The critical  $\alpha 2$ ,  $\beta 3$ , and  $\gamma 2$  subunits of GABA-A have been demonstrated to be present in human carotid bodies (Fagerlund et al., 2010).

Propofol has been demonstrated to depress AHVR in humans (Nagyova et al., 1995), as well as in the neural afferent response of the carotid body to hypoxia

(Jonsson et al., 2005; Akada et al., 2008). A number of pertinent mechanisms have been implicated, including depression of nicotinic signalling (pre- or post-synaptically) (Jonsson et al., 2005), voltage gated calcium channels (studied in smooth muscle) (Yamakage et al., 1995), or an influence on the CO<sub>2</sub>-O<sub>2</sub> chemosensory interactions (Akada et al., 2008). However, the effect of propofol on glomus cells has not been studied.

### **Nicotinic acetylcholine receptors**

Closely structurally related to the GABA-A channel, and part of the pentameric cis-loop ligand-gated ion channel family, the nicotinic acetylcholine channel (nAChR) plays a role in fast neuronal transmission. Many of the subtypes of this channel have been shown to be inhibited by anaesthetics including halothane (Mori et al., 2001), isoflurane, sevoflurane (Scheller et al., 1997) and propofol (Flood et al., 1997). The physiological consequence of this interaction is not just receptor inhibition, but also a decrease in neurotransmitter release (Westphalen et al., 2009), as nAChR receptors are also present pre-synaptically (Vizi and Lendvai, 1999), rendering fast-synaptic transmission vulnerable to modulation by anaesthetics.

In clinical practice, it is well known that curare-like neuromuscular blockers (e.g. vecuronium), which antagonise nAChR, obtund the AHVR (Eriksson et al., 1992; Eriksson et al., 1993; Eriksson, 1999). In animal models, this has been attributed to inhibition of neuronal transmission (Jonsson et al., 2004; Jonsson et al., 2006), though it is important to recognise that nAChR are present in the glomus cells of rats (Meza et al., 2012) and humans (Fagerlund et al., 2010). This pre-synaptic anatomical location implicates nicotinic mechanisms in the

autocrine and paracrine control of glomus cell excitation (Nurse, 2014), possibly allowing rapid amplification of relatively small magnitude changes in chemostimuli: akin to the classical exponential positive-feedback seen in many physiological processes.

### **Adrenoceptors**

Though the adrenoceptor family are metabotropic, and thus not suited to effecting fast synaptic transmission, they regulate a wide variety of ionotropic channels through G-protein coupled transduction (Alexander et al., 2013). Of specific interest in anaesthesia is the  $\alpha_2$  receptor subtype. Agonism of these receptors causes sedation in humans (Maze and Tranquilli, 1991; Belleville et al., 1992) and they have been implicated specifically in hypnosis in animals (Zhang et al., 2015). It is likely that they also have a role in analgesia (Lakhlani et al., 1997).

$\alpha_2$ -adrenoceptors are expressed pre-synaptically in many tissue types and species. Their role is predominantly thought of as regulating excitatory neurotransmission through presynaptic negative feedback. Noradrenaline has been demonstrated to inhibit the carotid body response to hypoxia (Suguihara et al., 1995), though it is difficult to separate the efferent and afferent innervation of the organ from the direct effect on the glomus cell and its vascular supply. Sympathetic modulation of carotid body chemosensitivity is variable and the location and subtype expression of adrenoceptors may be responsible for this.  $\alpha_2$ -adrenoceptors have been demonstrated by radio-binding assays on rabbit glomus cells (Kou et al., 1991).

A recent study showed that low-concentration dexmedetomidine facilitated the carotid sinus nerve activity in hypoxia (Nakatani et al., 2012). This represents the first report of a positive effect of dexmedetomidine on the ventilation chemoreflex, but also of significance is the lack of a demonstrable negative effect, even at relatively high doses.

### **Hypoxia physiology**

The physiological mechanisms that mediate the complex responses to hypoxia are the result of cellular and integrative processes, with a temporal range of milliseconds (glomus cells in the carotid body) to many days (erythropoetic response). Specific to the ventilation, there is an evolution of the mammalian response to hypoxia that develops from an inhibition of in utero respiratory movements by low oxygen tension, to the rapid and sustained stimulation of rate and depth of breathing in the adult.

### **Tissue responses to hypoxia**

Anatomically discrete tissues respond dissimilarly to hypoxia: the carotid body is excited and releases neurotransmitters, whereas the pulmonary vascular smooth muscle contracts to divert blood flow away from lung units that contain insufficient oxygen. Neuroepithelial bodies present in the lung epithelium secrete neurotransmitters in response to hypoxia, and may function to affect local changes in blood flow in response to low oxygen tensions. Although a slower response, the hypoxia-stimulated erythropoietin release represents a further adaptive physiological response to oxygen levels. By causing an increase in haemoglobin synthesis, the oxygen carrying capacity of the blood is increased and therefore delivery of oxygen to tissues improves.

The important distinction between these four mechanisms in hypoxia (carotid body, pulmonary smooth muscle, neuroepithelial bodies and erythropoetic response) and those in other non-oxygen-sensitive tissues is that each affects some degree of systemic protection against hypoxia. Whereas skeletal muscle for example will cease to function in hypoxia (a passive process), oxygen-sensing tissues have evolved to generate an active response to hypoxia. The resultant effect is remote (distant) to the sensor, recruiting other organs. It is also of critical importance that each of these tissue responses occurs within a plausible oxygen range and are therefore relevant in physiological homeostasis, rather than only being recruited in severe or extreme environments.

### **Pulmonary vascular response to hypoxia**

In contrast to the relaxant effect of hypoxia on the systemic vasculature, in the pulmonary arteries, hypoxia causes vasoconstriction (Motley et al., 1947). The pulmonary artery smooth muscle cells (PASMC) in their own right could be considered an effector of a discrete or intrinsic oxygen sensor. Discrete PASMCs contract in response to hypoxia (Murray et al., 1990) and as a result of membrane depolarisation, an increase in  $[Ca^{2+}]_i$  (Cornfield et al., 1994) occurs reminiscent of the glomus cell response to hypoxia.

### **Carotid body**

The carotid body is a highly specialised organ and is found consistently in the neck at the bifurcation of the carotid arteries in mammals. This organ has the highest blood flow to volume ratio yet demonstrated (Daly et al., 1954; Barnett et al., 1988), though it is likely much of this flow is shunted in euoxic conditions (Kumar and Prabhakar, 2011). In most species, the blood supply is derived from

the adjacent carotid artery, the primary purpose of which is to supply blood to the brain. It can be considered that this blood supply is representative of blood content (or gas tension) being supplied to the brain, and is therefore able to “detect” hypoxia or hypercapnia before the central nervous system is exposed to it. It is also consistent with the rapid breath-to-breath physiological sensory role of the carotid body (Barnett et al., 1988). At an ultrastructural level, the carotid body has a unique vascular structure to manage this exceptional blood flow, which also determines function. Multiple arteriovenous anastomoses (Acker 1980) and fenestrated capillaries adjacent to glomus cells (Biscoe 1966) have been implicated in chemosensory function. Shunts may cause “plasma skimming” towards glomus cells and divert erythrocytes though to the venous drainage (Acker and Lübbers, 1977), while fenestrated vessels passing in close proximity to glomus cells along convoluted paths (McDonald and Larue, 1983) allow efficient diffusion of respiratory gases .

Acute reduction in the blood flow to the carotid body does not cause an increase in chemoreceptor output, suggesting that it is the  $pO_2$  rather than bound oxygen content of blood that determines the magnitude of chemostimuli (Biscoe et al., 1970). In a pathophysiological correlate, the sustained reduction of carotid body blood flow observed in models of chronic heart failure have been shown to *augment* peripheral chemosensitivity (Ding et al., 2011). This serves to underline that local regulation of flow and regulation of systemic signalling pathways (e.g. renin-angiotensin-aldosterone) are also important in chemosensory function.

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It is now well established that the carotid body has significant afferent and efferent neural supply. The carotid sinus nerve (Hering's nerve) innervates the carotid body, itself a branch of the glossopharyngeal nerve. Ascending pathways of the carotid sinus nerve fibres terminate in the nucleus tractus solitarius, which is part of the dorsal respiratory group. The sensory nerve terminals are closely associated with glomus cells, often interconnected by reciprocal synapses (McDonald and Mitchell, 1975), which allow cell-cell communication and the formation of the classical hyperbolic pattern of discharge despite not all cells achieving afferent synaptic contact. Descending fibres can be identified by their expression of neuronal nitric oxide synthase and project not only to glomus cells (where their role is thought to be inhibitory (Campanucci and Nurse, 2007)) but also to the vasculature. There, NO elicits vasodilatation and an increase in blood flow (Wang et al., 1995). The net effect is that efferent innervation of the carotid body is autoinhibitory for chemoafferent discharge, and thus of great import in the plasticity of this physiological response.

Discrete of innervating and vascular tissue, the carotid body has two predominant cell types, designated type I (or glomus) and type II cells. Type I cell and glomus cell are terms considered interchangeable and are considered to be the chemosensory cells of the carotid body. These small, oval cells of ~10 µm diameter (in the rat), are most strikingly characterised by dense core vesicles, which contain secretory neurotransmitters, most notably catecholamines (Hansen et al., 1982).

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Surrounding clusters of glomus cells are enveloping type II cells. These cells number fewer and occupy significantly less volume of the carotid body than glomus cells. Historically, these have been thought of as purely sustentacular cells due to their lack of excitation by hypoxia (Lopez-Lopez et al., 1989). However, it has been demonstrated that purinergic receptors are present on type II cells and that ATP causes calcium influx (Xu et al., 2003) as well as further ATP release (Zhang et al., 2012) from these cells. This suggests a paracrine role for type II cells following type I cell activation by a chemostimulus.

Type II cells express markers of glial cells, and have been described as stem cells (Pardal et al., 2007). Recent evidence suggests that these cells may be activated by endothelin release from type I cells during chronic hypoxia (Platero-Luengo et al., 2014), stimulating organ growth. This fascinating mechanistic insight adds to the plasticity of the carotid body in the adaptive response to long-term hypoxia.

Human carotid body studies show agreement with the wealth of animal (particularly rodent) studies that have formed the foundation of understanding of the relationship between form and function. Particularly, recent work has confirmed the anatomical relationship of type I and type II cells (Ortega-Sáenz et al., 2013), that chemostimuli excite type I cell membranes (Ortega-Sáenz et al., 2013) and the hypoxia evoked neurosecretory function of glomus cells (Kåhlin et al., 2014). Of great importance, the demonstration of the presence of both the chemosensory apparatus (Mkrtchian et al., 2012) and evidence of the presence of anaesthetic sensitive elements (Fagerlund et al., 2010) in human

cells, supports the ongoing study of these interactions in animal cells which are more readily available and versatile.

### **Ventilatory responses to hypoxia**

The overall change in ventilation seen in hypoxia is time dependant, and many mechanisms contribute to this physiological response. Chronic hypoxic elicits significant plasticity in AHVR, the effects of which can be demonstrated after anything more than a five minute exposure to low oxygen tensions.

Prior to birth, mammals exhibit hypoxic inhibition of fetal breathing movements (FBM) (Bissonnette and Hohimer, 1987; Kobayashi et al., 2001a). These movements are important for maturation of the lung parenchyma (Harding, 1997). In utero, the carotid bodies respond as they do in adults: they are excited by hypoxia (Blanco et al., 1984). Denervating the carotid body afferent neural supply in utero obtunds the hypoxic inhibition of FBM. However, the significance of the hypoxic inhibition of FBM is unknown, particularly the role of the carotid bodies. FBM consume a large proportion (estimated to be ~30%) of fetal oxygen supply (Rurak et al., 1986), so during hypoxia increasing breathing movements (which have no capacity to offset the hypoxia) is deleterious. It is possible that the whole phenomenon is due to a neurohumoral (and protective) process which overcomes the peripheral chemoreceptor system during hypoxia. Fetal hypoxaemia drives catecholamine release (Hooper et al., 1990) and causes redistribution of blood through the ductus venosus to critical organs (Enhorning, 1993). Enhorning (1993) therefore postulated that stimulation of baroreceptors could mediate inhibition of FBM. This is plausible, and would

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account for the relatively rapid switch from hypoxic inhibition of breathing to stimulation soon after birth (Richardson et al., 2007).

The mature response to hypoxia is an initial increase in minute ventilation that is replicable across species (humans: Lindhard, 1911; Lloyd et al., 1958; rats: Aaron and Powell, 1993; mice: Kline et al., 2002), though the relative contribution of frequency and tidal volume are not (Frappell et al., 1992). This species variation may in part be due to the central nervous system processing of chemosensory input (Spyer and Gourine, 2009), and thus not directly a function of the carotid bodies.

There is a biphasic response to acute hypoxia in mammals (termed hypoxic ventilatory decline (HVD)) (Edelman et al., 1973; Bascom et al., 1990). Following an immediate increase in minute ventilation to ~100-150% of euoxic levels, over a further 5-10 minutes a slow decline to a new steady state is observed. The new plateau is arrived at approximately 15-20 minutes after onset of hypoxia (Powell et al., 1998), and typically is ~40% greater than pre-hypoxic minute ventilation (Teppema and Dahan, 2010). This “depression” is lasting, as after HVD is established and euoxic breathing restored, rechallenging a mammal with hypoxia will cause a zenith in minute ventilation of only ~50% greater than baseline (Easton et al., 1988). The anatomical site responsible for HVD has been contentious, with the contribution of depression of central respiratory drive (Neubauer et al., 1990) and alteration of the peripheral sensitivity to hypoxia (Robbins, 1995) debated. Species differences and the role of sleep and anaesthesia are in part to blame for the differences between studies. It is however primarily the peripheral site that is responsible

for this phenomenon, which was established using dynamic end-tidal forcing following sustained hypoxia in humans (Bascom et al., 1990). The subsequent sensitivity to hypoxia declines more than the fast component (the carotid body contribution) of the CO<sub>2</sub> ventilatory response (Bascom et al., 1990). This observation can only arise peripherally, unless neural signals from the carotid body are selectively encoded for different stimuli (which is unlikely).

Next in the temporal sequence of adaptation comes ventilatory adaptation to hypoxia (VAH), where exposure to hypoxia results in a sustained rise in ventilation (Rahn and Otis, 1949; Howard and Robbins, 1995). This occurs over hours to days in a milieu of hypoxia. This again is in part a carotid body phenomenon, as when the CSN is sectioned, VAH does not occur (Olson et al., 1988).

### **Cellular hypoxia**

Of course, all normal cells in anoxia will eventually die, and this could be argued to be an extreme form of hypoxic chemosensation, though clearly not an adaptive response. It is in the normal physiological range that an oxygen sensor must function. In the search for this sensor, a broad dichotomy has arisen between the metabolic (or mitochondrial) hypothesis and the membrane hypothesis (where ion channel mechanisms are key). Although these can be dissected into discrete mechanisms each independently exhibiting oxygen sensitivity, it is likely that multiple mechanisms contribute to a “chemosome”, where the constituent parts interact. Therefore, the graded nature of metabolic processes and fast temporal characteristics of ion channels combine to cause a ventilatory response that is both rapid and sensitive over a physiological range.

**Mitochondria**

Lack of sufficient intracellular oxygen perturbs normal mitochondrial electron transport chain flux in all cells. ATP production decreases and superoxide species are produced (Li and Jackson, 2002). These reactive oxygen species (ROS) damage nucleic acid and proteins and in combination with ATP deficiency, apoptosis may occur. Single cells can convert superoxide to hydrogen peroxide ( $H_2O_2$ ) through superoxide dismutase.  $H_2O_2$  therefore becomes a transducer of hypoxia as a physiological oxidizer (Hoshi and Heinemann, 2001). A number of mechanisms regulating cellular excitation in the carotid body such as BKCa are redox dependant (Wang et al., 1997).

Specifically in the carotid body, an early reconciliation of the mitochondrial hypothesis of oxygen sensing was proposed by Mills and Jobsis (1970), who determined that cytochrome a3 (complex IV) reduction occurred concurrent to chemoreceptor discharge from the carotid body of cats. They suggested that carotid body mitochondria cytochromes may have a lower affinity for oxygen than those found in other cells: an adaptation to become oxygen sensitive. The adaptation of these proteins to hypoxia appeared to be a limitation in  $V_{max}$ , without a change in  $K_m$ , akin to the way a non-competitive inhibitor might be described pharmacologically.

The exquisite sensitivity of mitochondria to oxygen tension in a physiological range was then demonstrated in rabbit glomus cells by Duchen and Biscoe (1992), using a combination of mitochondrial poisons (disrupting different points of the electron transport chain) and rhodamine 123 fluorescence quenching to assess mitochondrial membrane potential. It was initially postulated that this

caused an increase in  $[Ca^{2+}]_i$  due to release of mitochondrial stores of calcium. Though the sensitivity to mitochondrial poisons was reproduced later in rats (Buckler and Turner, 2013) and mice (Turner and Buckler, 2013), it is now established that it is extracellular calcium entry that follows mitochondrial depolarisation.

To date, no evidence exists to suggest this is an epiphenomenon, i.e. that there is an intermediate step between hypoxia occurring and glomus cell mitochondria depolarising. The stark contrast between glomus cells and control (usually superior cervical ganglion) cells in this respect supports the mitochondrial hypothesis of oxygen sensing due to its graded response over a plausible physiological range.

#### ***Hypoxia-inducible factor***

With anything more than transient hypoxia, a transcriptional signalling cascade is activated by hypoxia-inducible factor-1 (HIF-1) in most mammalian cells (Schofield and Ratcliffe, 2004). The stabilisation of HIF-1 occurs instantaneously in hypoxia, and is degraded upon restoration of oxygen supply with a half-life of 1-5 minutes (Semenza, 2004). Several hundred mRNAs have been demonstrated to be upregulated by HIF-1 during hypoxia (Semenza, 2011).

#### ***AMPK***

During cellular stress, a rise in the AMP/ATP ratio causes an activation of AMP-activated protein kinase (AMPK). This enzyme is exquisitely influenced by intracellular AMP concentration (Hawley et al., 1995), so is considered a metabolic sensor. Downstream effects of AMPK include cellular uptake of

glucose (Abbud et al., 2000) and an increase in fatty acid oxidation (Thomson et al., 2008) among a myriad of processes beneficial to a cell under metabolic stress (Kahn et al., 2005). Hypoxia can be considered as a significant cellular stress, and it has been hypothesised that low oxygen tension can activate AMPK in glomus cells (Wyatt et al., 2007). In this context, it is also of significant interest that AMPK regulates both BKCa (Ross et al., 2011) and TASK (Dallas et al., 2009) channel activity, potentially uniting the metabolic and membrane hypotheses.

### ***Gasotransmitters***

There are at least three gaseous signalling molecules that have been implicated in hypoxic signalling in the carotid body: nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H<sub>2</sub>S) (Prabhakar and Peers, 2014).

Of these, NO is produced constitutively in the vasculature of the carotid body by endothelial nitric oxide synthase (eNOS), and in fibres of the glossopharyngeal nerve by neuronal nitric oxide synthase (nNOS). The role of NO seems to be in descending inhibition of carotid body excitability through inhibition of voltage gated calcium channels (Summers et al., 1999) and activation of a calcium dependant potassium conductance (Silva and Lewis, 2002). Chronic hypoxia upregulates an inducible form of the enzyme producing NO (iNOS), and has been implicated in the blunting of chemosensitivity following chronic hypoxia.

As well as a near universal depression of AHVR across drug classes, anaesthetics also generally cause relaxation in smooth muscle, in common with endothelium-derived relaxing factor, a synonym for NO. Both volatile and intravenous anaesthetics tend to promote the release of NO from both

endothelial (eNOS) and neuronal (nNOS) sources (Toda et al., 2007), and though no NO-producing apparatus has been demonstrated in glomus cells of humans or mice (Mkrtchian et al., 2012), in an intact chemoreflex, this could be a mechanism by which anaesthetics enhance descending inhibition of chemosensitivity.

### **Ion channel mechanisms of hypoxia sensing**

It is accepted that a wide range of ion channels exhibit changes in conductance in response to hypoxia. Potassium, sodium, calcium and non-specific cation channels have all been implicated. Determining whether these conductance changes are as a direct result of hypoxia, or indirect regulation by an adjacent oxygen sensor has proved challenging, and at times controversial.

### **Potassium channels**

#### ***Kv***

The first report of an oxygen sensitive potassium current, also reported voltage-dependence (Lopez-Barneo et al., 1988). The hypoxic inhibition of Kv channels has been described not only in chemoreceptors, but also in HEK cells (Pérez-García et al., 1999), neuroepithelial cells (Youngson et al., 1993), vascular myocytes (Cox, 2005) and pulmonary smooth muscle cells (Weir and Archer, 1995). The great molecular diversity of the Kv channel family may account for variation in oxygen sensitivity between and within species and the heterogeneity across cell types and expression systems. This variation calls into question their role as an oxygen *sensor*, as in many cases, oxygen sensitivity can be demonstrated in their absence, and even when present, Kv channels are not always sensitive to hypoxia.

Consistency is found in how Kv channels influence resting membrane potential (RMP). Where Kv channels can be demonstrated to have an effect on RMP, for instance in rabbit carotid body glomus cells (Pérez-García et al., 2000) or pulmonary artery smooth muscle cells (PASMC) (Archer et al., 1998), they can also be linked to the initiation of the hypoxia transduction cascade. In systems where RMP is not influenced by Kv, for instance rat carotid body glomus cells, inhibition of these channels may contribute to the maintenance of membrane depolarisation in hypoxia once initiated by a discrete sensor.

### ***BKCa***

Early reports of a hypoxia inactivating calcium-sensitive potassium current (BKCa) in glomus cells (Peers, 1990a) suggested a key role for this conductance in determining the cell membrane response to low oxygen tension. The most marked effect of hypoxia in these experiments was in the  $K^+$  current over the test potential range -10mV to +50mV with a maximal effect at +20 mV. This does not in itself implicate BKCa in the initiation of depolarisation as glomus cells were subsequently shown to have a resting  $E_m$  (membrane potential) of -59 mV (Buckler, 2012), far below the typical activation threshold of -50 mV to -40 mV for glomus cell BKCa (Ganformina and Lopez-Barneo, 1992). However, the large conductance of these channels could result in a contribution to maintaining  $E_m$ , even if the open probability is low under resting conditions. If this were the case, selective pharmacological block of these channels (e.g. by tetraethylammonium) would influence the current-voltage relationship, which it does not (Buckler, 1997). This stands true for isolated cells, though when opposed to other glomus or sustentacular cells in co-culture or tissue slices, many estimates of  $E_m$  are more positive (Stea and Nurse, 1991; Abudara and

Eyzaguirre, 1998), making interpretation of the role of BKCa in the intact carotid body difficult to predict from isolated cell experiments (Peers and Wyatt, 2007). Regardless, BKCa has a significant role in the maintenance of depolarisation.

### *K<sub>2P</sub>*

The discovery of a voltage- and time-independent hypoxia sensitive potassium current added a third biophysically distinct potassium channel to those that were already known to influence glomus cell chemoexcitation (Buckler, 1997). This potassium conductance remains the most plausible for *initiating* membrane depolarisation in hypoxia as it is active at the RMP of glomus cells. Pharmacological inhibitors of BKCa and Kv do not influence this background conductance. The molecular identity of the background potassium conductance was advanced by the observation that the biophysical characteristics of a then recently cloned member of the K<sub>2P</sub> family of channels, TASK 1 (Fink et al., 1996; Duprat et al., 1997), resembled that of the background potassium conductance of rat glomus cells (Buckler et al., 2000). The suggestion that at least part of the background conductance is sensitive to changes in extracellular [Mg<sup>2+</sup>] (Williams and Buckler, 2004) yielded TASK 3 as functionally significant in the initiation of hypoxia induced membrane depolarisation. By comparing native channels to concatameric TASK 1 and 3 constructs (Kim et al., 2009) and analysing channels in cells isolated from TASK 1 and 3 single and double knockout mice (Turner and Buckler, 2013), the TASK 1/3 heteromultimer has been established as the predominant oxygen sensitive background potassium conductance of the rat glomus cell.

Though TASK channels are routinely oxygen sensitive in native cells, in expression systems there is equivocation. Both TREK 1 (Miller et al., 2003) and TASK 1 have been reported to be oxygen sensitive in HEK293, but these results have not been reproduced (Buckler and Honore, 2005). Even when interrogating native channels, there is a dependence on the intracellular milieu (possibly MgATP) to maintain the oxygen sensitivity of background potassium conductance (Varas et al., 2007). As such, though well placed as early in the transduction of hypoxia signalling, there is likely to be an upstream sensor.

### ***HERG***

The human ether a go-go gene encodes for the HERG channel. Although its name suggests it is canonically sensitive to anaesthetic (ether), this to a certain extent is a conflation. It is the fly gene “ether a go-go” which when mutated causes ether induced movement disorders (Warmke and Ganetzky, 1994). However, there is evidence of HERG-like currents in glomus cells (Overholt et al., 2000), and contributes to the resting membrane potential, and therefore excitability of these cells. Activation of this current was sufficient to cause chemoafferent discharge from the carotid body. In cardiomyocytes HERG has been extensively investigated as a cause for arrhythmogenesis. In the course of these investigations halothane, which frequently causes dysrhythmia when administered, was shown to inhibit HERG currents (Li and Correa, 2002). Therefore, although not directly oxygen sensitive, by virtue of the effects of HERG on resting membrane potential in glomus cells, and the prospect of its inhibition by halothane, this channel may contribute to the depression of glomus cell hypoxic excitation by anaesthetics.

### **Calcium channels**

Voltage-gated calcium channels ( $\text{Ca}^{2+}_v$ ) are undoubtedly an important step in hypoxic stimulus transduction. The hypoxia induced increase in  $[\text{Ca}^{2+}]_i$  is dependent on extracellular  $\text{Ca}^{2+}$ , and voltage-clamping glomus cell membranes attenuates this increase (Buckler and Vaughan-Jones, 1994a). L- and T-type  $\text{Ca}^{2+}_v$  blockers also attenuate the hypoxia induced catecholamine secretion in glomus cells (Cáceres et al., 2009). There is some evidence that volatile anaesthetics inhibit  $\text{Ca}^{2+}_v$  (Joksovic et al., 2005), and this is manifest as a modest reduction in potassium evoked increase in glomus cells (Pandit and Buckler, 2009; Pandit et al., 2010b).  $\text{Ca}^{2+}_v$  have been shown to be invulnerable to intravenous agents in a variety of models (Hall et al., 1994; Orestes and Todorovic, 2010).

### **Sodium channels**

The presence of a significant voltage-gated sodium conductance in rabbit glomus cells (Lopez-Barneo et al., 1988) is not mirrored in other species including rat (Stea and Nurse, 1991), excluding this as a driving part of the generation of action potentials. In order to balance the background potassium efflux present in glomus cells, a reciprocal cation influx is thought to be present (Buckler, 1997). Consistent with this, in the absence of extracellular sodium glomus cells hyperpolarise (Carpenter and Peers, 2001). However, recent evidence (Kang et al., 2014) has added a non-selective calcium activated sodium conductance to the depolarisation sequence of glomus cell membranes. It is not constitutively active, seems to be dependent on  $[\text{Ca}^{2+}]_i$  and is only recruited in severe hypoxia. As such, though this channel could significantly influence the magnitude of cellular response to hypoxia, it is unlikely that its

absence would abolish chemosensitivity, and in itself could not initiate depolarisation.

### **Could $K_{2P}$ channels mediate the anaesthetic depression of AHVR?**

Of great significance in the mechanistic review presented here, is that the only unitary mechanism that has consistently proven to be both oxygen and anaesthetic sensitive is the  $K_{2P}$  channel family TASK. The parsimony of this explanation for the anaesthetic depression of acute ventilatory responses in cells and animals is elegant. However, even if a definitive causal link could be established, it is likely that a number of factors, including histological, anatomical and temporal all contribute to what is a highly specialised and adaptive protective response. This thesis will attempt to establish a greater degree of understanding of the interaction between anaesthetics and single channels, cells and animals in the context of hypoxia.

### **Thesis objectives**

Based on the work of others, it is accepted that the carotid body mediates the anaesthetic depression of AHVR, at least in part. Anaesthetic agents will act differently from each other at the carotid body, and by establishing their actions on the peripheral chemosensor, rational combinations of hypnotic drugs or the use of channel blockers may ameliorate the general depressive nature of anaesthetics on AHVR.

As such, my thesis has the following objectives:

1. In order to determine whether TASK channels are responsible for the volatile depression of AHVR, first, the effect of genetic ablation of TASK channels on the ventilatory response to hypoxia and hypercapnia in mice

- will be assessed. The chemosensitivity of these animals in the presence of anaesthetics will give an insight into the role of TASK channels in mediating anaesthetic depression of AHVR. This is explored in Chapter 3.
2. To examine whether propofol will directly depress the carotid body glomus cell response to hypoxia, I will measure the effect of this drug on calcium influx in neonatal rat type I cells. If it does indeed depress the chemosensitive unit of the carotid body, the mechanism by which this occurs will be explored using electrophysiology and pharmacological manipulation of putative targets. This is addressed by Chapter 4.
  3. As dexmedetomidine has been proposed to augment the carotid body response to hypoxia, I will assess whether when isolated from surrounding neural tissue, this effect is preserved. I will also assess the effects of sub-hypnotic doses of dexmedetomidine on unstrained mouse AHVR, which is presented in Chapter 5.
  4. To further understand the role of  $K_{2P}$  channels in initiating glomus cell excitation by hypoxia, I will quantify the effect of specific pharmacological antagonism of TASK 1 and TASK 3 on glomus cell intracellular calcium. If blocking these channels can independently cause glomus cell excitation, I will assess whether their presence augments cellular responses to hypoxia, and whether blocking TASK can restore cellular oxygen sensitivity. This investigation forms Chapter 6.

**Chapter 2:**

**GENERAL METHODS**

### **Isolation of carotid body glomus cells**

The function of carotid body has been studied since in detail since the 1920s (Nobelstiftelsen; Heymans and Bouckaert, 1930), and over the subsequent decades, an increasingly reductive approach to the study of this organ occurred. From the early detailed anatomical descriptions of de Castro in the 1920s (de Castro, 2009), came an understanding that glomus cells were closely opposed to nerve fibre endings (De Kock, 1954) and were likely to contain catecholamines (Lever et al., 1959). These neural signals were soon being studied and it was demonstrated that in isolation, the carotid body was a polymodal chemosensor (Eyzaguirre and Lewin, 1961). Pietruschka (1974) was the first to successfully isolate carotid body cells for study in culture, work which ultimately led to the discovery of oxygen and acid sensitive ionic currents (Lopez-Barneo et al., 1988; Peers, 1990b; Buckler, 1997) and chemostimuli induced calcium influx into type I glomus cells (Buckler and Vaughan-Jones, 1993; Buckler and Vaughan-Jones, 1994a; Buckler and Vaughan-Jones, 1994b).

### **Excision of carotid bifurcations**

The most extensively studied species in the field of isolated glomus cells is the rat. For the experiments presented here, the Sprague Dawley rat was used, as it is the most widely used outbred strain and therefore lends itself to comparative study.

Animals are operated on between postnatal days 10-14 (P10-14), as beyond this age fibrous tissue proliferates and makes microsurgery on the delicate vascular structures surrounding the carotid body challenging. Equally, once

excised, isolation of glomus cells requires digestion of this fibrous tissue, a process that if too prolonged or severe can damage the chemosensory function of type I cells.

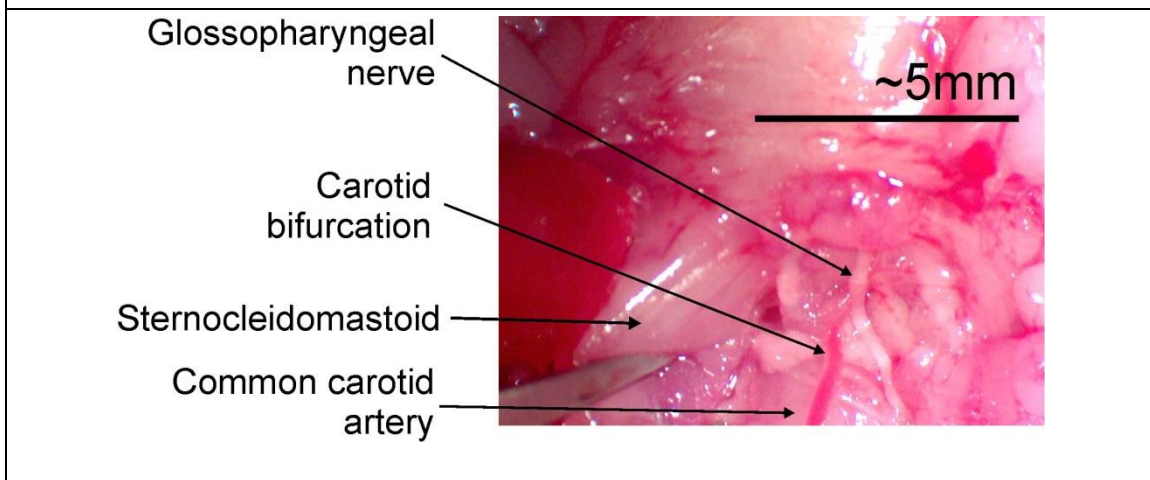
There is however a post-natal developmental window, where animals transition from the blunted in utero hypoxic responses to that of a mature adult. Many mammals demonstrate hypoxic inhibition of breathing movements during gestation (Boddy et al., 1974; Kobayashi et al., 2001b), though peripheral the carotid body is responsive to hypoxia (Blanco et al., 1984), suggesting significant central inhibition of this chemoreflex. It is probably due to the emergence of the characteristic O<sub>2</sub>-CO<sub>2</sub> synergy that accounts for this change with aging (Pepper et al., 1995), a phenomenon that is thought to be mediated by the peripheral chemoreceptors. In rats, the maturation of hypoxic response is complete by P8 when measured in isolated cells or by carotid sinus nerve recording (Bamford et al., 1999).

### ***Surgery***

Animals were anaesthetised in an induction chamber with of 2-4% isoflurane in air, prior to transfer to a low volume nose-cone and breathing circuit, maintained at a flow rate of 0.5-1 l.min<sup>-1</sup>. In the supine position, the upper limbs were fixed by taping in extension. After ensuring a surgical plane of anaesthesia by abolition of the pedal withdrawal reflex, a skin flap was raised from the anterior neck exposing tissues from the level of the clavicles inferiorly to the hyoid superiorly. Following blunt retraction of the salivary glands and division of the sternomastoid inferiorly, the course of the carotid artery from its emergence into the neck to its bifurcation was traced. Dissection of tissues surrounding the

bifurcation was achieved using fine forceps, with care taken to expose the two branches of the carotid artery away from the bifurcation (Figure 2.1). Once free from surrounding tissue, a silk suture ligated the carotid artery low in the neck. Immediate division of the branches and common carotid artery meant only a second or two passed from warm perfusion of the carotid body to cooling in Dulbecco's phosphate buffered saline (PBS). This minimised the warm ischaemic time of this highly metabolically active organ. The process was repeated on the contralateral side, prior to decapitation of the animal while under terminal anaesthesia. Typically two animals were used (four carotid bodies) on a day of experiments.

**Figure 2.1** Operating microscope view of the left neck dissected in situ. The pertinent anatomy is labelled; the common carotid is ligated before excision of the bifurcation *en bloc* and immediate immersion in ice cold Dulbecco's phosphate buffered saline (PBS).

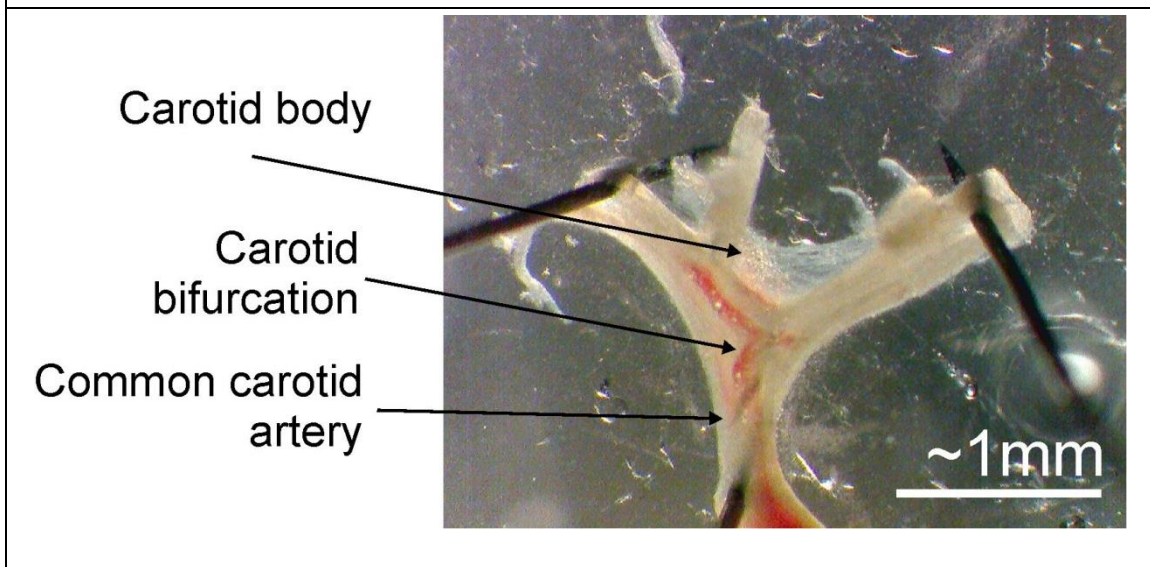


### ***Subdissection***

While still in ice-cold PBS, each carotid bifurcation was mounted on a silicone elastomer plate (Sylgard, Dow Corning) in the anatomical position using 100  $\mu$ m stainless steel Minutien pins (Figure 2.2). The plate has superior optical qualities to allow transillumination and fine dissection of excess fibrous tissue.

The superior cervical ganglion often overlies the carotid body, and is characteristic in its larger size and “fatty” texture when handled. It was gently peeled away and discarded. Once identified as in Figure 2.2, the carotid body was excised using fine Vannas scissors and kept in a separate iced PBS dish.

**Figure 2.2** Ex vivo dissection of the carotid body from its adjacent structures. The optical qualities of the Sylgard mounting plate allow transillumination of the structures while immersed in PBS. The carotid body is identified by its anatomical position and characteristic pearlescent appearance in this light. Often, a branch nerve leading to the glossopharyngeal nerve can be identified.



#### ***Enzymatic treatment***

Once all carotid bodies were excised, they were pooled and placed in an enzyme solution containing 0.6 mg/ml type I collagenase (Worthington, Lakewood, USA) and 0.4 mg/ml trypsin (Sigma Aldrich) in warmed Ham's F12 (Gibco). This dish was kept at 37°C for 15 minutes prior to gentle teasing of the intact carotid bodies to increase surface area for digestion. After a total of 22 minutes in the enzyme solution, the carotid bodies were removed and washed first in modified cell culture medium (Table 2.1), before transfer into the same solution, additionally containing 250 µg/ml trypsin inhibitor (Sigma Aldrich).

**Table 2.1** Constituents of primary cell culture medium for dissociated glomus cells, prepared at most 72 hours in advance.

Ingredient	Volume	Final concentration
Nutrient mixture F12 in		
Dulbecco's Modified Eagle Medium (F12/DMEM)	50 ml	90.1%
Fetal bovine serum	5 ml	9.0%
L-glutamine (mg/ml)	500 $\mu$ l	0.9%
Insulin (10 mg/ml)	20 $\mu$ l	0.04%

#### ***Mechanical disruption of carotid bodies***

Mechanical dissociation of the partially digested carotid bodies occurred by trituration using fire polished Pasteur glass pipettes (VWR, Radnor, USA), with an approximate internal tip diameter of 125  $\mu$ m to 370  $\mu$ m as measured by wire. Through repetitive suction into and expulsion from the capillary tube of the pipette, the tissue was dispersed over approximately 5-7 minutes, under direct microscopic vision.

This cell suspension (~30  $\mu$ l) was pipetted onto 6 mm diameter glass coverslips (VWR) coated with poly-L-lysine 0.01% (Sigma Aldrich).

#### **Microspectrofluorimetry**

##### **Overview**

It has long been recognised that calcium is essential for the excitation of tissues. Indirect evidence first came from the seminal work of Ringer (1883) in demonstrating that calcium was required in the perfusate of hearts to sustain

normal contractility. It took almost 80 years for the report of calcium's effect on neuronal tissue to emerge (Heilbrunn, 1940) which emphasised that intracellular calcium was the link between membrane depolarisation and cellular effects. But it was not until the 1960s that reliable methods of measuring intracellular calcium were described (Ridgway and Ashley, 1967). Since the original account of the calcium-activated bioluminescent protein aequorin (Shimomura et al., 1962), the vast majority of studies on the biological role of intracellular calcium concentration have exploited fluorophores. A major refinement arrived with the synthesis of the calcium sensitive dyes Indo-1 and Fura-2 by Grynkiewicz et al. (1985). The importance of these tools was recognised with Nobel Prizes for Shimomura and Tsien for their advancement in the imaging of cells by fluorescence techniques.

### **Measuring calcium concentration in glomus cells**

Prior to the widespread use of microspectrofluorimetry to quantify  $[Ca^{2+}]_i$  in glomus cells, it had been established that hypoxia caused an influx of calcium using radiolabelled  $^{45}Ca$  (Pietruschka and Acker, 1985) This provided the causal link between hypoxia and catecholamine release from glomus cells that had earlier been observed (Fidone et al., 1982).

Biscoe and Duchon (1990) demonstrated in rabbit glomus cells that  $[Ca^{2+}]_i$  was increased by hypoxia using Fura-2 and asserted that the predominant source was a mitochondrial store being released. Rat glomus cells were analysed by Buckler and Vaughan-Jones (1994a) and found the contrary: that Indo-1 fluorescence ratio changes (and so  $[Ca^{2+}]_i$ ) was dependant on extracellular calcium ( $[Ca^{2+}]_o$ ), and its transport across the membrane by  $Ca^{2+}_v$  channels.

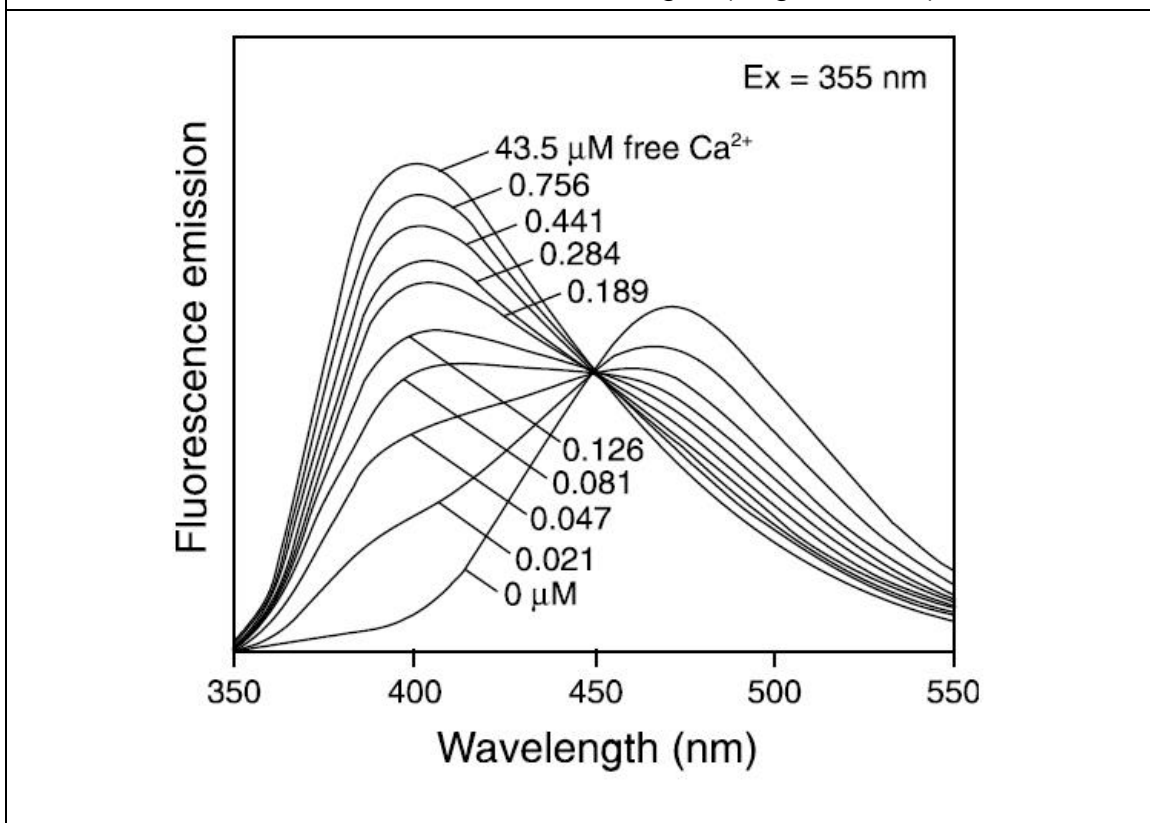
This established the use of fluorescent dyes in the study of chemostimulation of the carotid body.

Indo-1 has been used in its acetoxymethyl (AM) ester form for all of the studies presented here. Using the AM form of the compound allows efficient uptake of the fluorophore, in a calcium insensitive state. It is only activated after intracellular esteratic cleavage to render Indo polar and therefore sensitive to calcium (Figure 2.3). As with older dyes such as Quin2, buffering of  $[Ca^{2+}]_i$  can occur in higher concentrations. To avoid problems with this and accumulation in organelles, the lowest concentration possible is used (2.5  $\mu$ M), and in this regard Indo-1 is less likely to become organelle confined than Fura-2 (Wahl et al., 1990). Indo-1 has the advantage of a higher  $K_d$  for calcium than Fura-2, allowing a superior temporal resolution of changes in  $[Ca^{2+}]_i$  (Jackson et al., 1987). High concentrations are required when the excitation or emission spectra overlap with cell autofluorescence spectra. This is a key concern in hypoxia sensing, as the  $NAD^+/NADH$  ratio is affected by the influence of hypoxia on the electron transport chain. Both Indo-1 and Fura-2, can be illuminated with less excitation intensity than older dyes such as Quin2, as they have a greater fluorescence emission intensity. This avoids NADH autofluorescence (which requires significant illumination intensity) and also reduces "photobleaching". Indo-1 however does not require mechanical excitation light switching, so benefits from a simpler instrumentation to illuminate cells.

Ratiometric methods have significant advantages over the use of single wavelength dyes. As any change in the fluorescent intensity at one wavelength increases, at the other there is decrease, enhancing the change that would be

observed at a single wavelength. Therefore there is amplification of the signal. As signal changes are always dual, uneven loading of the dye throughout the cell has an internal control, avoiding the need for cell by cell calibration. Similarly, the path length (through cells/tissue of varying dimensions) and intensity of illumination do not need to be controlled specifically. Therefore, Indo-1 AM is the superior  $[Ca^{2+}]_i$  indicator, as it has the greatest signal-to-noise profile, while minimally perturbing the cell with illumination light.

**Figure 2.3** Emission spectra of indo-1 in a solution containing a range of  $[Ca^{2+}]$  concentrations. The calcium sensitive peak emission occurs at 405 nm for and the calcium insensitive peak at 450 nm. In this example, excitation is with light  $\lambda=355$  nm. From Molecular Probes Inc. catalogue (Eugene, USA)



### Loading with dye

After two hours allowing the glomus cells to adhere to poly-L-lysine coated cover slips, the dish containing the coverslips was flooded with 2 ml F12/DMEM

medium adding 5  $\mu\text{g}$  Indo-1 AM for a final Indo concentration of 2.5  $\mu\text{M}$ . A further incubation in air with added 5%  $\text{CO}_2$ , at room temperature in darkness was carried out for 60 minutes to allow de-esterification. The coverslips were then removed from the Indo solution and place them in fresh F1/DMEM until use, to avoid “overloading” the cells with dye.

### **In vitro calibration of $[\text{Ca}^{2+}]_i$**

In order to calibrate fluorescence emission ratios of Indo-1 to known  $[\text{Ca}^{2+}]_i$ , glomus cells were rendered calcium permeant with the ionophore ionomycin. At a concentration of 10  $\mu\text{M}$  ionomycin, calcium can pass freely between the extracellular superfusate and the cytoplasm. Following normal loading with Indo-1 AM dye (above), cells were further incubated in a  $[\text{Ca}^{2+}]$ -free buffer (In mM: 140 KCl, 20 HEPES, 11 Glucose, 1  $\text{MgCl}_2$ , 10 EGTA, pH 7.4 at 37°C). Ethylene glycol tetra-acetic acid (EGTA) was used as a calcium chelating agent. After 30 minutes, glomus cells were identified microscopically, and sequentially superfused with the same  $[\text{Ca}^{2+}]$ -free buffer as above, representing the  $R_{\min}$   $[\text{Ca}^{2+}]_i$ , and with 10 mM  $[\text{Ca}^{2+}]$ -HEPES buffered solution (EGTA omitted), as  $R_{\max}$ . These ratios were averaged and used to calibrate  $[\text{Ca}^{2+}]_i$  in subsequent experiments (Equation 2.1).

### **Rig setup**

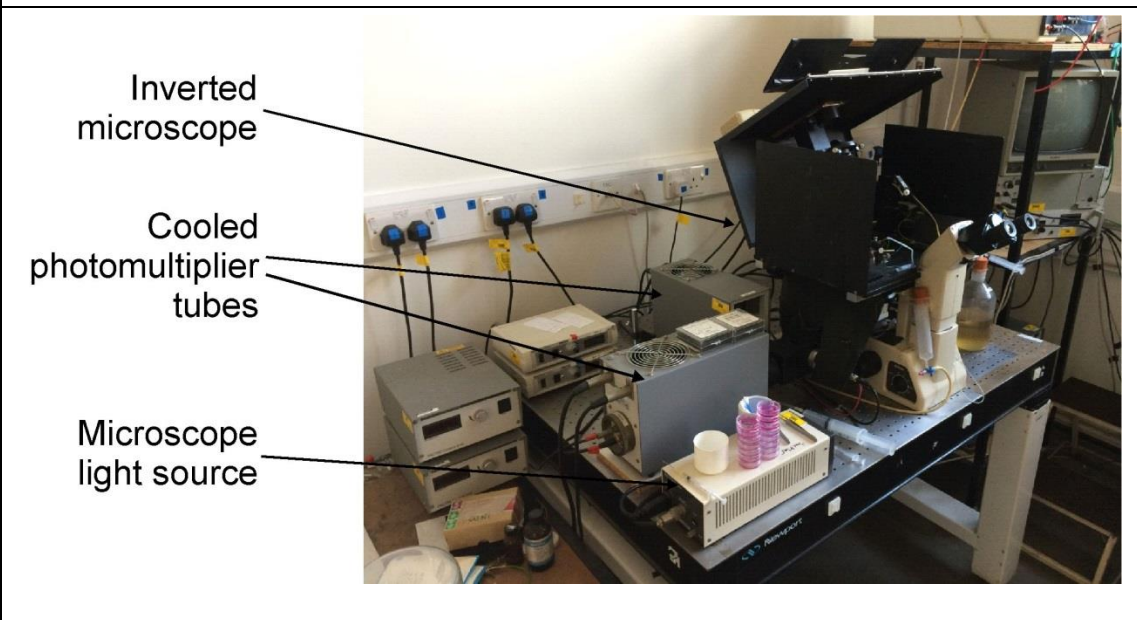
The system for visualising and measuring the fluorescence from glomus cells consists of an inverted microscope (Nikon Diaphot) and a pair of cooled photomultiplier tubes (Thorn EMI, London, UK) coupled to an arc lamp by a series of dichroic mirrors and band pass light filters (All Nikon, Figure 2.4 and Figure 2.5). The excitation bandpass filter had centre a centre wavelength at

340 (bandwidth 15) nm, emission bandpass filters at 405 (43) nm and 495 (20) nm. Dichroics were longpass at 390 nm (camera) and 450 nm (between PMTs). The superfusion bath was constructed of perspex, with a coverslip glass bottom. The total volume of the measuring chamber is 100  $\mu$ l.

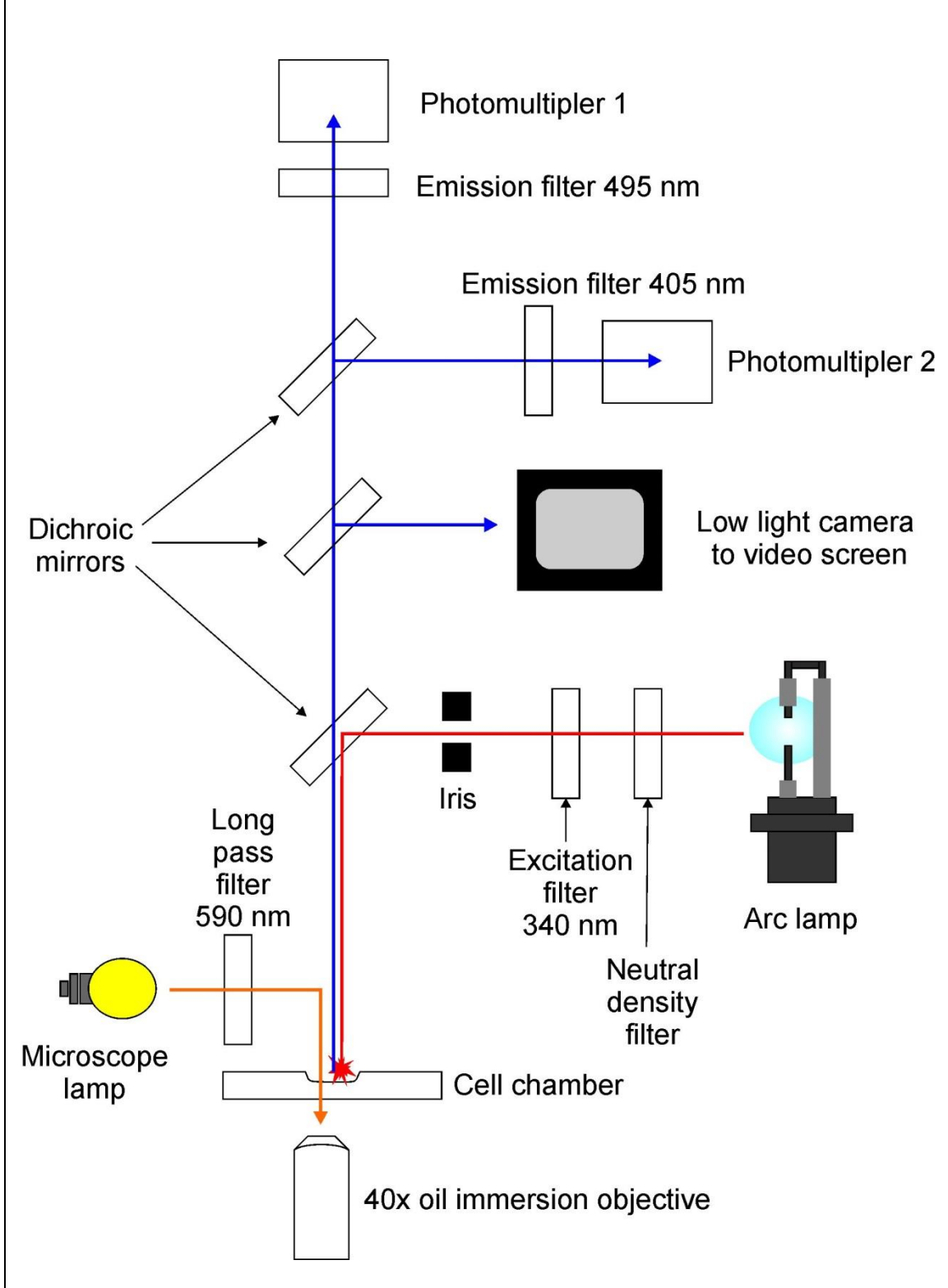
**Equation 2.1** Where  $R$  is the measured fluorescence intensity ratio at 405 nm to fluorescence at 495 nm,  $R_{min}$  is the fluorescence intensity ratio of indo-1 when unbound to calcium,  $R_{max}$  the fluorescence ratio of calcium-bound indo-1 and  $F_{495,free/bound}$  the ratio of the free to bound indo-1 at 495 nm.  $K$  is the dissociation constant for  $Ca^{2+}$  of indo-1, which is generally taken as 230 nM (Bassani et al., 1995)

$$[Ca^{2+}]_i = K_d + \left( F_{495, \frac{free}{bound}} \right) \cdot \frac{R - R_{min}}{R_{max} - R}$$

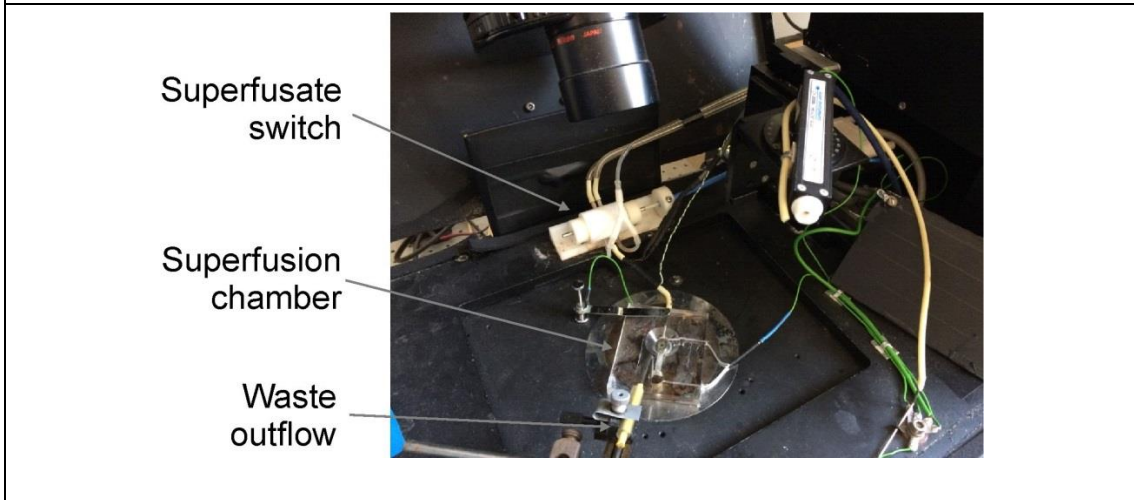
**Figure 2.4** Fluorescence microscopy, fluorimetry and electrophysiology rig. The housing for the photomultiplier tubes are to shield from light and maintain a temperature of -18 to -20 °C to reduce interference. The microscope platform is also shielded from light and all equipment sits on an air table to minimise movement artefact. The arc lamp is not visible in this image.



**Figure 2.5** Diagram of inverted fluorescence microscope setup. For Indo-1 dye, the maximal excitation wavelength is 340 nm. The emission peak sensitive to calcium is 405 nm. At 495 nm, the emission spectra is insensitive to free-calcium concentration. In this diagram, red lines represent excitation light, and blue is the emitted fluorescent light.



**Figure 2.6** Superfusion bath. The total volume of the bath is ~100  $\mu$ l. The reference and earthing electrodes for patch clamp electrophysiology can be seen leading to both measurement and waste chambers. Also visible is the mechanical superfusate switch represented in **Figure 2.8**.



### Delivering chemostimuli

In order to create electrolyte solutions with varied gas compositions, glass bottles containing Tyrode's solution (Table 2.2), typical volume 100-1000 ml, were warmed in a water bath and certified gas mixtures (British Oxygen Company, Surrey, UK) bubbled into them using gas dispersion tube (Corning, New York, USA). Superfusate was drawn from the gas-equilibrated solutions through stainless steel tubing to the cell chamber (Figure 2.6) by gravity and then removed from the outflow by a peristaltic vacuum pump.

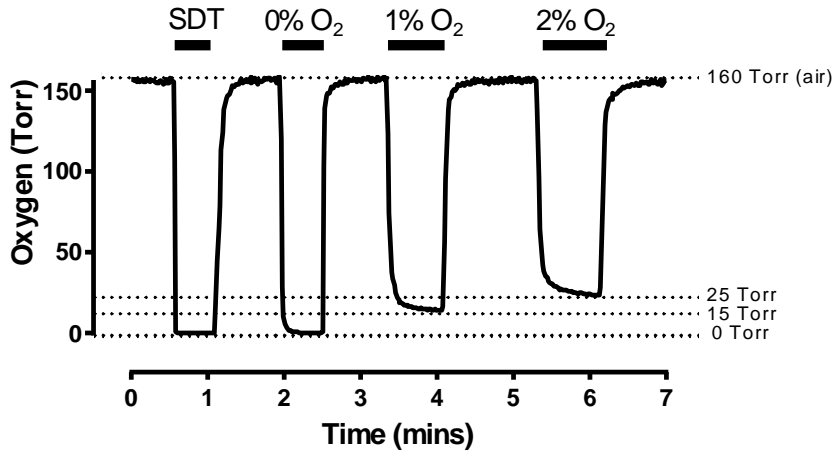
**Table 2.2** Tyrode's solution, pH 7.4 when equilibrated with 5% CO<sub>2</sub> containing gases.

<b>Ingredient</b>	<b>Final concentration</b>
KCl	4.5 mM
NaCl	117 mM
NaHCO <sub>3</sub>	23 mM
CaCl <sub>2</sub>	2.5 mM
MgCl <sub>2</sub>	1 mM
Glucose	11 mM

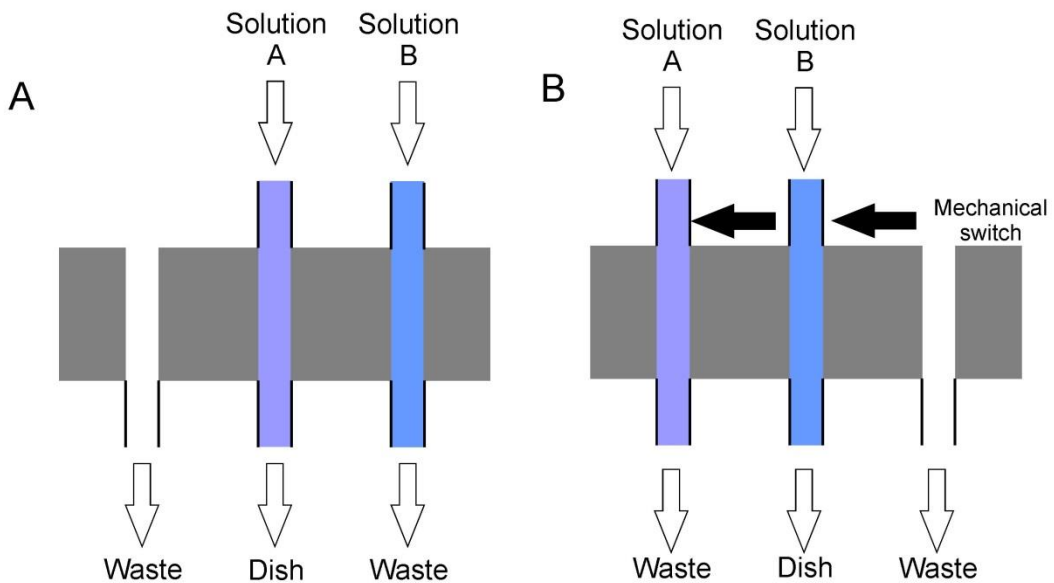
The superfusate being delivered to the chamber was switched using a custom made set of taps (Figure 2.8), with minimal dead space.

Oxygen tension in the bath can be varied between 0-160 Torr reliably. This is demonstrated in Figure 2.7. This illustrates not only the ability to rapidly deliver a robust hypoxic chemostimulus without chemical oxygen scavengers, but also that the system does not entrain, diffuse or leak oxygen.

**Figure 2.7** Measurement of oxygen tension in cell superfusion chamber using a fluorescence quenching optode (Presens). SDT is Sodium Dithionite, and oxygen scavenger, which creates a chemically anoxic environment. Graded hypoxia is shown such that 0% O<sub>2</sub> is 0 Torr, 1% O<sub>2</sub> is 15 Torr, and 2% O<sub>2</sub> is 25 Torr.



**Figure 2.8** Diagram of the rapid switching mechanism to superfusion dish. The solution of interest always runs through the central channel. In panel **A**, the cells in the dish are being bathed in solution A. Mechanically switching the inflow (panel **B**) allows a rapid change to a different milieu with a total volume change occurring in under 10 seconds. Diagram based on schematic drawings by Mark Howard.



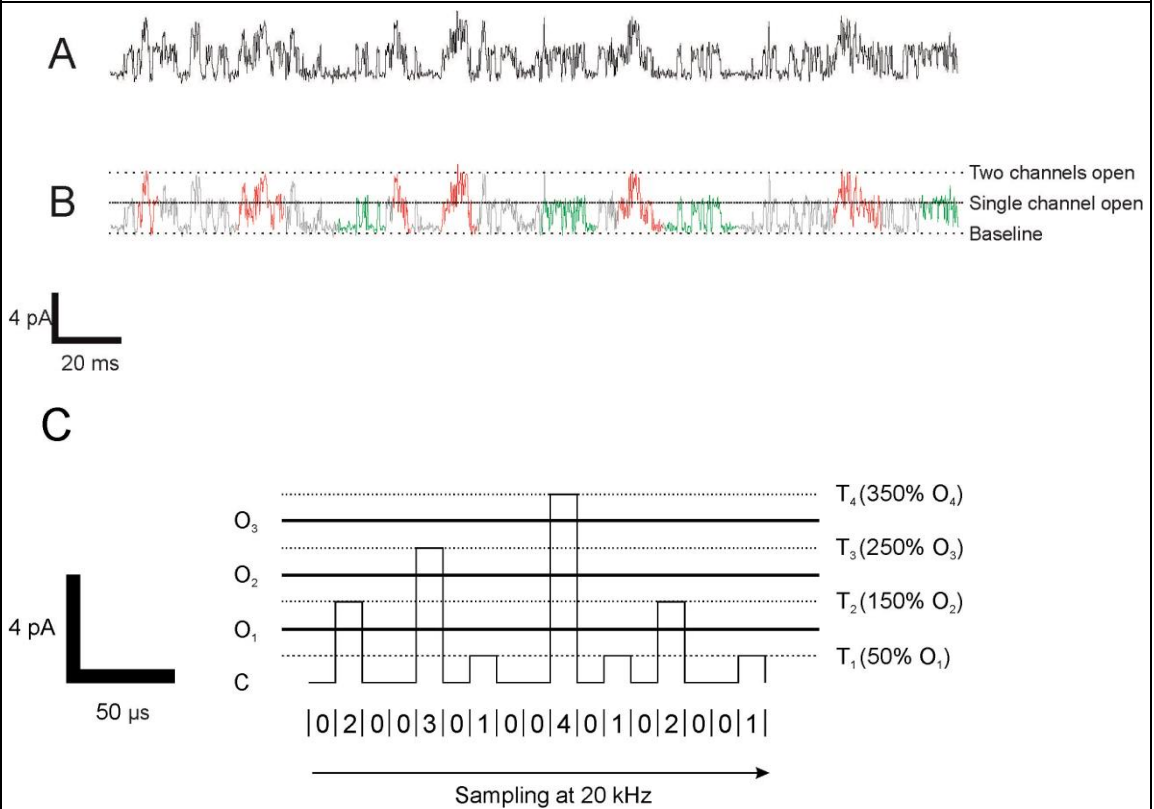
### **Patch clamping**

For analysis of TASK channel activity, cell-attached patch clamp electrophysiology was performed. Using borosilicate glass capillaries coated with Sylgard (Dow Corning, Seneffe, Belgium) and fire-polished immediately before use. Cell-attached filling solution contained 140 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM HEPES, 10 mM tetraethylammonium and 5 mM 4-aminopyridine at pH 7.4 at 37°C. Recordings were conducted in 100 mM KCl and a pipette potential of +80 mV. The signal was filtered at 2 kHz and current recorded at 20 kHz prior to data acquisition with a CED 1401 analogue to digital converter and Spike2 software (CED, Cambridge, UK). The main conductance state for each recording was defined using an all points histogram, and the threshold for opening set at 50% of this value. Multiple openings were defined as current of 150% of the main conductance state. As multiple channels were often present in a patch, single-channel activity is therefore presented as  $NP_{\text{open}}$  (where total observed channel open time is indexed to the number of channels in the patch) and compared before and during application of propofol.

### ***Quantifying TASK channel activity***

As multiple channels are often observed in membrane patches, and multiple channels can open simultaneously, it is important to index total TASK conductance for an estimate of the number of channels present. TASK channels have flickery kinetics, and may have multiple open and closed states. The technique I used to quantify channel activity was  $NP_{\text{open}}$ , and is explained in Figure 2.9.

**Figure 2.9 A.** Typical TASK channel trace **B.** the same trace, for clarity, examples of double channel openings (red) and single channel openings (green). **C.** A simplified representation of a digitised channel recording. To calculate  $NP_{open}$ , initially, it is necessary to define the amplitude of a single channel opening ( $O_1$ ) from an all-points histogram. The threshold for a single opening is set below this level, in this example 50% ( $T_1$ ). Multiple openings are subsequently defined as 150%, 250%, etc., of this threshold. When sampled at 20 kHz, each quantum of data is assigned a “multiple” of the threshold (or 0 when below  $T_1$ ). The average value therefore becomes  $NP_{open}$ . If it were possible to definitively count the channels ( $n$ ) in the recorded patch,  $P_{open}$  (the probability that any channel is open at any given time), would be defined as  $P_{open} = \frac{NP_{open}}{n}$ .  $NP_{open}$  quantifies total channel activity, indexed for an estimation of the number of channels present, whereas  $P_{open}$  can only assess the net effect of a single channel type.



In this example:

$$NP_{open} = \frac{0 + 2 + 0 + 0 + 3 + 0 + 1 + 0 + 0 + 4 + 0 + 1 + 3 + 0 + 0 + 1}{17} = 0.88$$

## **Measuring ventilatory responses in rodents**

### **Overview**

A standard approach to quantifying the AHVR in humans is described by the “Lake Louise proposal” (Powell, 2006) which was the culmination 30 years of debate since early attempts to create consensus (Kronenberg et al., 1972; Severinghaus, 1976). In the intervening time, it became possible to accurately and reproducibly control the end-tidal CO<sub>2</sub> tension (ETCO<sub>2</sub>) in humans through rapid servo-controlled gas switching with respiratory gases measured using mass spectrometry (Robbins et al., 1982a; Robbins et al., 1982b). This established “dynamic end-tidal forcing” (DEF) as the pre-eminent tool (Teppema and Dahan, 2010) to measure not just the AHVR, but also to create a controlled stimulus to measure the pulmonary vascular response to hypoxia (Smith et al., 2008) and the cerebral response to CO<sub>2</sub> (Wise et al., 2007).

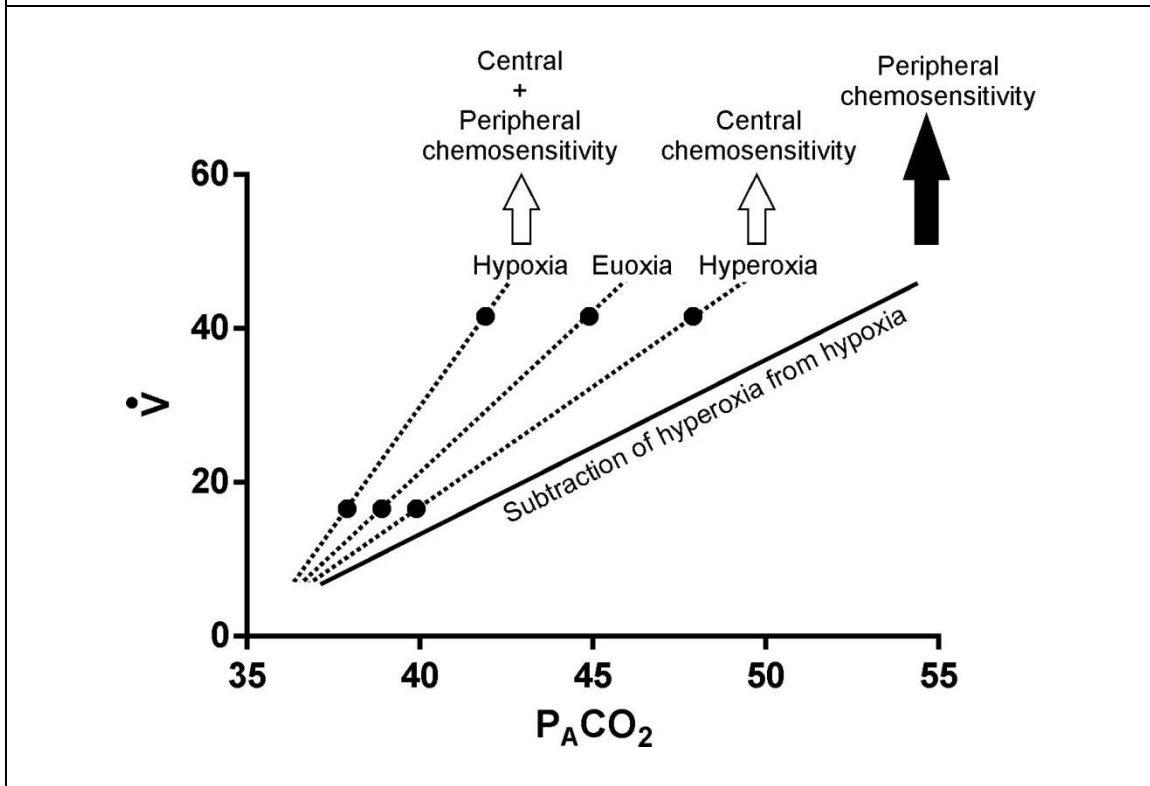
In rodents, this is not possible. With ventilatory rates of 200-400 breaths per minute is not feasible to measure ETCO<sub>2</sub>, let alone dynamically change the inspire accordingly. As such, a different paradigm must be sought.

In the “Lake Louise Proposal”, the ability to clamp and relinquish control of CO<sub>2</sub> allows quantification of both isocapnic and poikilocapnic hypoxic responses. The poikilocapnic response can be readily measured by standard plethysmographic techniques (described later, see: Unrestrained plethysmography), but the isocapnic response is more challenging. As hypoxia effects an increase in ventilation ( $\dot{V}_E$ ), a greater amount of CO<sub>2</sub> is excreted, and the PaCO<sub>2</sub> is lowered. This in itself *reduces* carotid body stimulation, and  $\dot{V}_E$  will tend towards the pre-hypoxic level. As this is a dynamic response, no steady-

state is achieved; the magnitude of AHVR is small and unlikely to be reproducible in the face of depressant or stimulants of ventilation.

Prior to the invention of DEF, Cunningham and Lloyd (1963b) had described a nuanced approach to removing this confounder. By measuring the AHVR over a range of inspired  $\text{CO}_2$  and  $\text{O}_2$  concentrations, the influence of hypercapnia can be mathematically separated from that of hypoxia (Figure 2.10). This was advanced in a modern context by Teppema and Dahan (2010), who suggested that by measuring  $\dot{V}_E$  across a range of step changes in inspired  $\text{O}_2$  and  $\text{ETCO}_2$ , the  $\text{O}_2$ - $\text{CO}_2$  interaction could also be described.

**Figure 2.10** Representation of the relationship between  $P_A\text{CO}_2$  and ventilation ( $\dot{V}$ ) By quantifying ventilation in step changes into a range of inspired  $\text{CO}_2$  and  $\text{O}_2$ , the slope of the relationship represents sensitivity to hypoxia, and a subtraction can be carried out to calculate the contribution of the peripheral chemoreceptor. After Cunningham and Lloyd (1963a) and Duffin (2007)



The use of super-physiological levels of O<sub>2</sub> (hyperoxia) has been said to “silence” the peripheral chemoreceptor (Dejours, 1962) . This principle has also been used to study the contribution of the peripheral chemoreceptor in intact mice (Kline et al., 2002), where genetic mutation may perturb the chemoreflex at multiple points in the reflex arc.

Based on these protocols, this thesis will describe the ventilatory response in the context of step changes into both pure hypoxia, with no available inspired CO<sub>2</sub>, and euoxia, hypoxia and hyperoxia with variation of inspired CO<sub>2</sub>, as a proxy for ETCO<sub>2</sub>.

### **Unrestrained plethysmography**

Direct methods of measuring respiratory mechanics in animals required intubation of the trachea, for which an anaesthetic must be delivered for compliance of the subject. Anaesthetics have an independent effect on ventilation and chemosensory responses, and are indeed the subject of this thesis. Therefore, as a dependent variable in my experiments, delivering anaesthetics for their *clinical* endpoints is confounding, both as I intended to avoid general anaesthesia and as the tests to measure depth of anaesthesia constitute either pain or arousal. Therefore, the method of choice is one in which the animal is not restrained (by means of a cuffed collar) or instrumented (for instance with a tracheostomy).

Early indirect quantitation of  $\dot{V}_E$  (often in neonatal humans) was heavily based on sealing a chamber of fixed volume around a subject's neck (Boutourline-Young and Smith, 1950) . By measuring the pressure change in the box, a calibrated tidal volume could be inferred. Although less invasive, the level of

## *Chapter 2 - General Methods*

restraint is itself potentially confounding, and for welfare reasons would likely require sedation. It is also fraught with error, given reliance on a seal around the neck.

Following the work of Drorbaug and Fenn (1955), a barometric method of measuring respiratory volumes was derived. This is based on the warming and humidifying effect of the respiratory system on inspired gases. Cool, dry inspired gas is warmed and humidified by the airways, resulting in an increase in its volume. This causes a rise in the pressure within the chamber. Epstein and Epstein (1978) refined the earlier equations to take into account that chamber pressure rise ( $P_1$ ) during inspiration is not equal to pressure fall ( $P_2$ ) during expiration, as the chamber warms and ambient humidity increases. In fact,  $P_1/P_2$  is calculated to equal  $\sim 2$ . It is Epstein's equation that modern unrestrained plethysmography is based on (Equation 2.2).

The magnitude of acute ventilatory response to any chemostimulus was defined as in Figure 2.11.

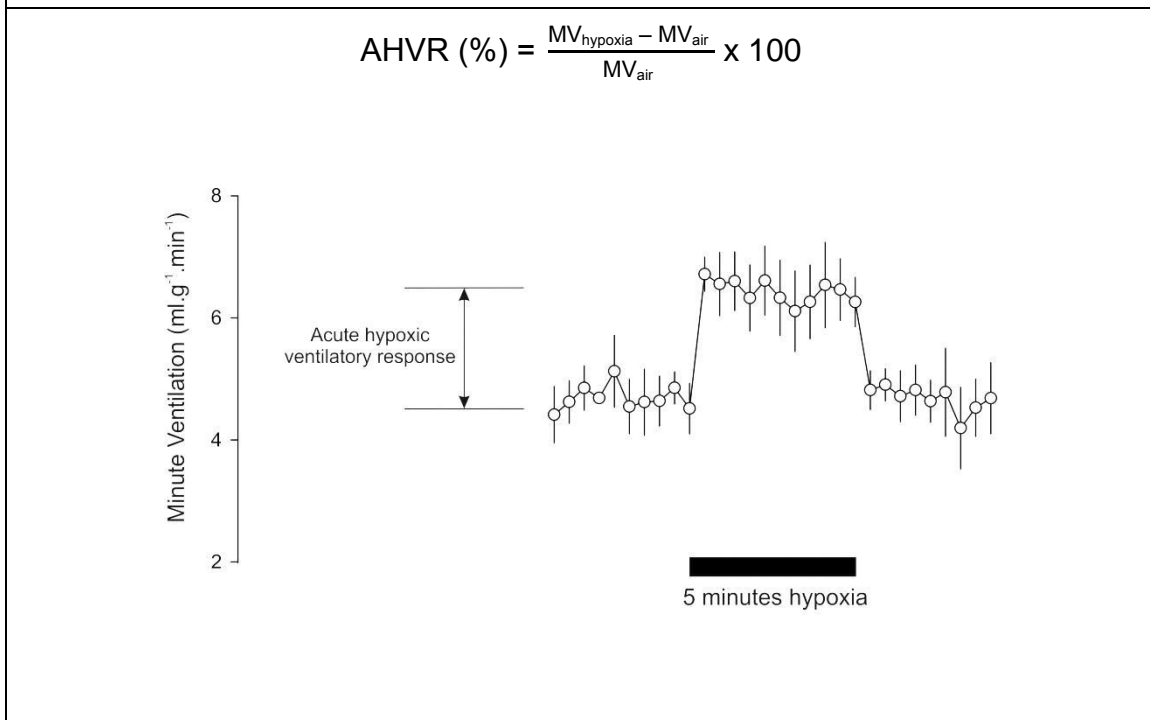
**Equation 2.2** Equation to determine tidal volume from pressure change in plethysmography chamber

$$V_T = A \frac{Vk}{Pk} \cdot \frac{T \cdot (P_a - p)}{T \cdot (P_a - p + A) - t \cdot (P_a - P + A)} - S$$

[Modified from Drorbaug and Fenn (1955)]

where  $V_T$  is tidal volume (ml),  $A$  is pressure increase during inspiration (Torr),  $Vk$  (ml) is the volume injected into the chamber to calibrate and  $Pk$  (Torr) is the pressure change due to  $Vk$ .  $T$  is alveolar temperature (assumed to be constant @ 310°K),  $P_a$  (Torr) is the barometric pressure in the chamber,  $P$  (Torr) is the partial pressure of water in the alveolus (assumed to be 37 Torr), and  $p$  (Torr) is the partial pressure of water in the chamber (as fresh dry gas is constantly flushed, this is 0 Torr).  $S$  is the correction applied by Epstein and Epstein (1978).

**Figure 2.11** Calculating the acute ventilatory response. In this example, the stimulus is hypoxia, but the quantification is the same for all stimuli. The AHVR is normalised to the baseline (euoxic) minute ventilation, to account for potential changes in this when TASK channels are perturbed.



### **Defining an optimal single isocapnic stimulus for C57BL6 mice**

Whole body plethysmography was performed by placing a conscious mouse in a plexiglas chamber (Figure 2.12), which was continually flushed with gas mixtures at a flow rate of  $2 \text{ l}\cdot\text{min}^{-1}$ . This flow rate was described by measuring the gas exchange time of the chambers, using a zirconia-based fuel cell  $\text{O}_2$  analyser (Rapidox 3100X, Cambridge Sensotec, Cambridge, UK) (Figure 2.15).

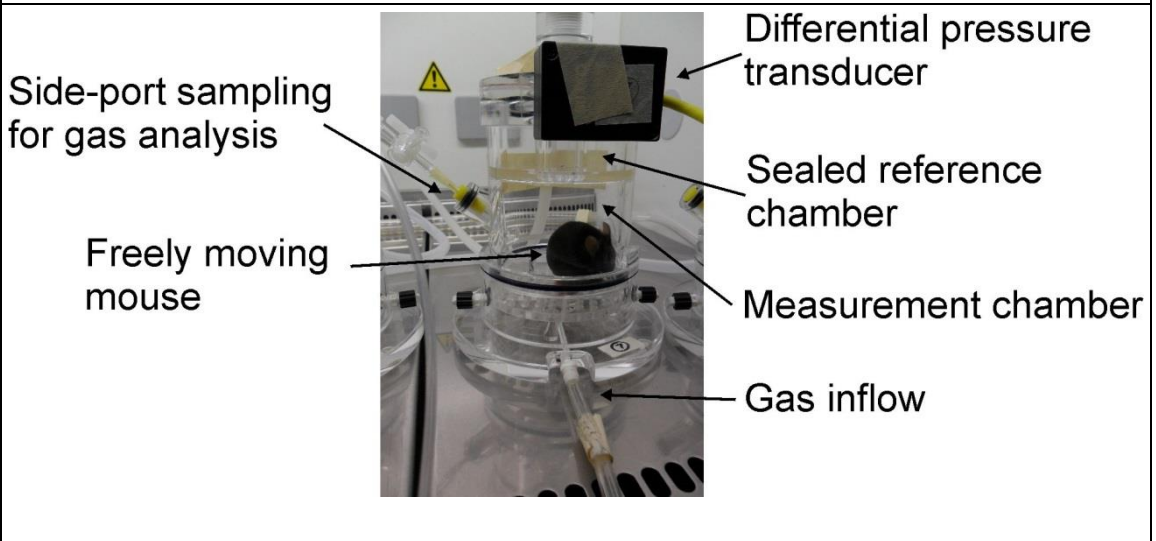
Pressure changes were measured with a differential pressure transducer, the signal amplified, filtered and converted analogue-to-digital through a preamplifier (Buxco Electronics, Wilmington, NC) (Figure 2.14). Signal processing was performed by proprietary Finepointe software (Buxco).

To ensure that the derivation of minute ventilation and respiratory rate of this proprietary software was both consistent and accurate, raw pressure data were extracted, and for a cohort of animals dual analysed independently using Labchart (AD Instruments, Dunedin, New Zealand).

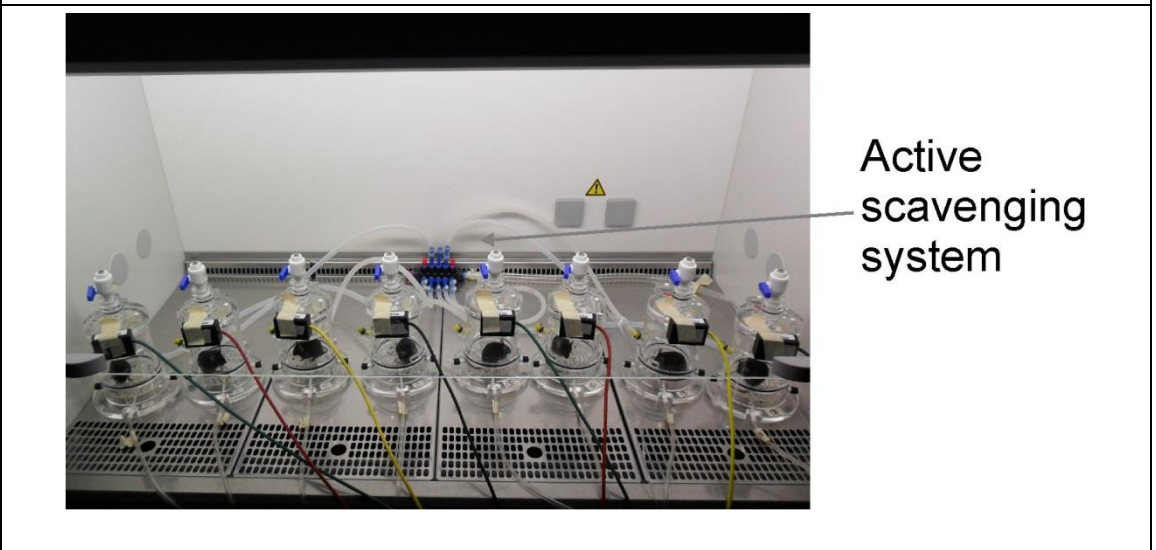
Animals were acclimatised to the chambers for a period of 30 minutes prior to experimentation.

To establish the optimal “isocapnic” hypoxic stimulus in C57BL6 animals, preliminary studies were carried out. Over the full range of  $\text{O}_2$  concentration (10-21%) with 0-4%  $\text{CO}_2$ , the minute ventilation during 5-minute step changes from air into each gas mix was measured. The results are presented in Figure 2.16.

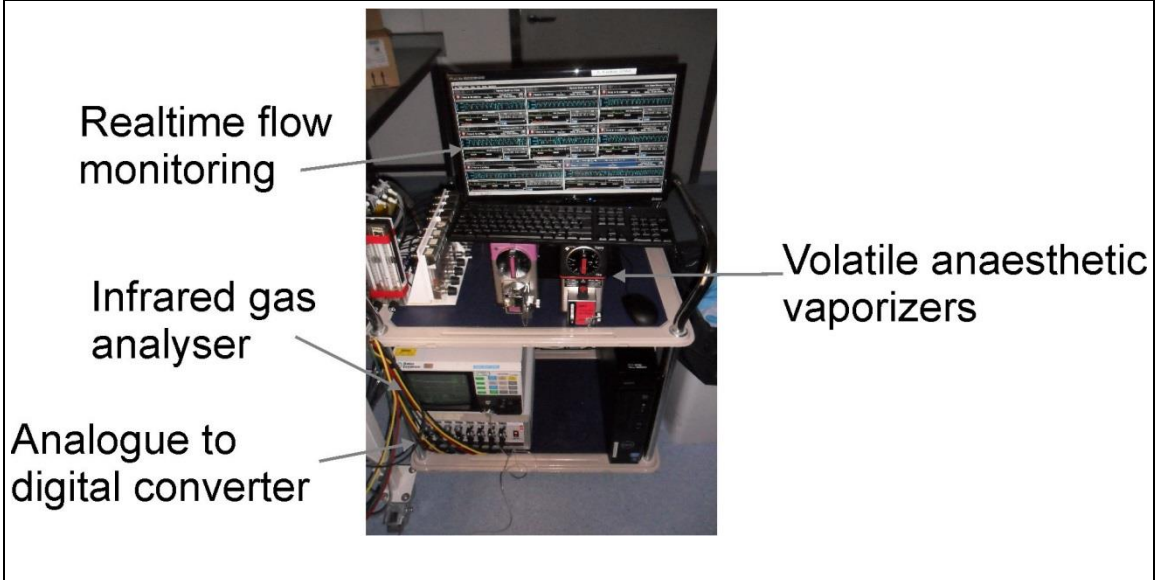
**Figure 2.12** Unrestrained whole body plethysmography chamber. Each mouse is housed in a sealed Perspex chamber. Calibration occurs prior to an animal being sealed in.



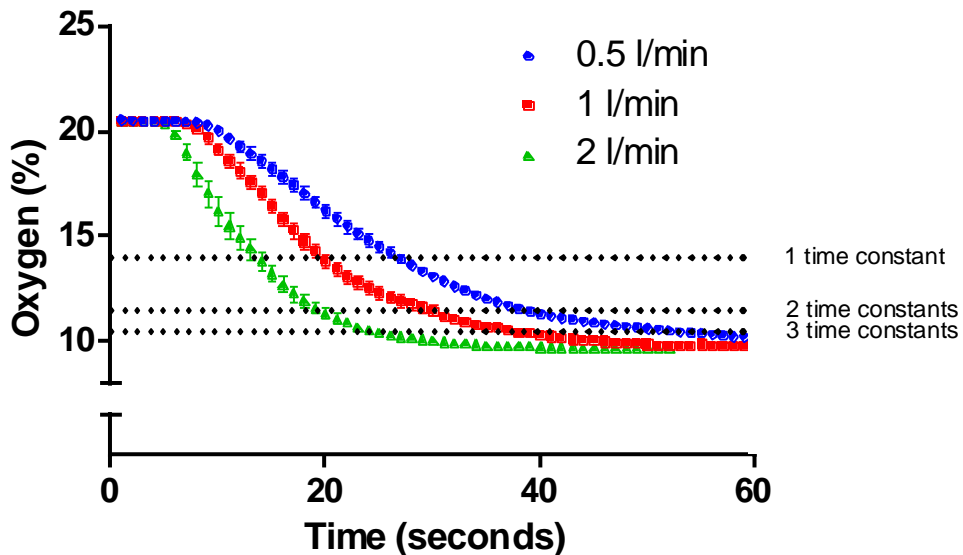
**Figure 2.13** A wider view of animals during plethysmography. Each chamber has a gas flowmeter controlling the flow rate, after splitting of the output from a master flowmeter and vaporizers. Given the total flow of >10 litres per minute, each chamber has active scavenging of the waste gas expelled from the chamber.



**Figure 2.14** Data processing equipment and volatile anaesthetic delivery system.



**Figure 2.15** Measuring the gas exchange time of plethysmography chamber. With 8 technical replicates for each of the three flow rates 0.5, 1 and 2 litres/minute, the concentration of oxygen in the chamber was measured using mass spectroscopy. Gas switch from 21% O<sub>2</sub> to 10% O<sub>2</sub> occurs at time=0seconds. As can be seen, a near total exchange of gas occurs in under 30 seconds with a flow of 2 l.min<sup>-1</sup>, yet for 0.5 l.min<sup>-1</sup>, this does not occur until almost 60 seconds. Data are presented as % ± SEM.



### Quantifying hypoxic ventilatory decline

Although HVD was not the primary outcome measure of the ventilatory studies in mice, it was quantified regardless. The AHVR was first measured as demonstrated in Figure 2.11, by quantifying the peak  $\dot{V}_E$ . A second value for  $\dot{V}_E$  measured at the end of the hypoxic exposure and the difference between this and the peak value determined. By expressing this as a proportion of the AHVR, a greater value indicated more hypoxic ventilatory decline (Equation 2.3).

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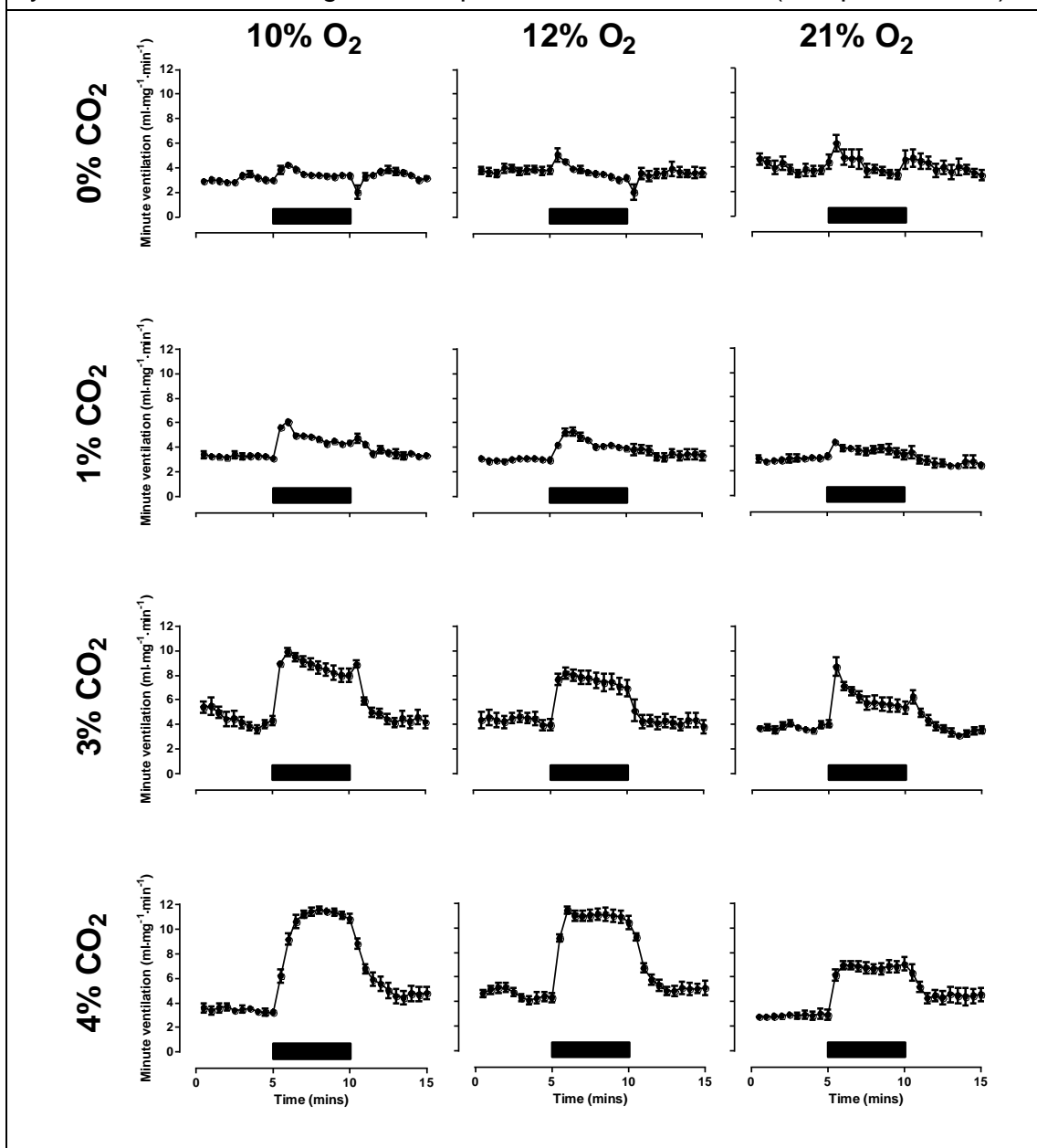
**Equation 2.3** Calculation of hypoxic ventilatory decline

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$$HVD (\%) = \frac{\text{Peak } \dot{V}_E - \text{End hypoxic } \dot{V}_E}{AHVR} \times 100$$

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**Figure 2.16** Defining an optimal isocapnic stimulus (see text for further detail). This array represents the change in minute ventilation to a variety of inspired gas mixtures (period of hypoxia black bar). Increasing CO<sub>2</sub> concentration top to bottom. This highlights a number of important observations: (1) The response to poikilocapnia is small or absent; (2) the magnitude of response to 1% CO<sub>2</sub> across the range of O<sub>2</sub> concentrations is relatively small and produces little by way of a graded response between 10% and 12% O<sub>2</sub>. This suggests that these two stimuli are unlikely to maintain isocapnia. Then: (3) In 4% CO<sub>2</sub>, post-hypoxic ventilation does not return to pre-hypoxic baseline values on return to euoxia, which suggests this stimulus is too high (i.e., an ongoing acid/CO<sub>2</sub> chemostimulus) and represents hypercapnia rather than isocapnia. It is only with 3% CO<sub>2</sub> stimulus that a characteristic “square wave” response to hypoxia is seen, reminiscent of the human response to isocapnic hypoxia when using dynamic end-tidal forcing. All data points are mean ± SEM (n=8 per stimulus).



### Blood gas analysis

To ensure that the proposed isocapnic hypoxic stimulus (10% O<sub>2</sub> with 3% CO<sub>2</sub> in balance N<sub>2</sub>) was successfully offsetting hyperventilation alkalosis, I conducted blood gas analysis on C57BL6 blood at the end of a 5-minute exposure to this gas.

Four animals were used, with a control group of a further four animals which breathed air in the plethysmograph for the same duration. Following cervical dislocation while still in the experimental environment, the total blood volume of each animal was aspirated from the great vessels of the neck into a heparinised syringe. Blood gas analysis was conducted at the point of experiment (GEM 3000, Instrumentation Laboratory, Warrington, UK), with less than 10 seconds from exsanguination to analysis.

There was no significant difference in the CO<sub>2</sub> tension (p=0.139) or the pH (p=0.916) between the two groups (Table 2.3).

**Table 2.3** Blood gas analysis comparing isocapnic hypoxia with air.

<b>Ambient gas</b>	<b><i>Isocapnic hypoxia</i></b>	<b><i>Air</i></b>	<b><i>p-value</i></b>
pH	7.38±0.01	7.35±0.02	p=0.916
pCO <sub>2</sub> (kPa)	5.00±0.09	5.73±0.19	p=0.139

This establishes 10% O<sub>2</sub> with 3% CO<sub>2</sub> in balanced nitrogen as the optimal isocapnic stimulus for C57BL6 animals.

Volatile anaesthetics were delivered to the chambers by integrating plenum style agent specific vaporizers into the gas delivery system prior to individual

chamber rotameters (Figure 2.14). Infrared gas analysis was conducted at multiple points along the delivery system to ensure no losses of gas and monitored continuously at the point of delivery to the animal (Figure 2.12).

### **Quantifying post-hypoxic breathing behaviour**

The C57BL6 mouse, the background genotype of the genetic knockout models presented here, has been extensively studied with regard to breathing patterns and post-hypoxic and post-hypercapnic breathing behaviour (Getsy et al., 2014). They display many of the traits that are considered normal in humans, such as the pulmonary arterial response to hypoxia. However, it is of note that after exposure to low oxygen concentrations their breathing behaviour is dominated by post-hypoxic frequency decline (PHFD), where breathing frequency falls below the baseline. It is considered “normal” that in physiological terms this is balanced by short-term potentiation (STP), and this imbalance is feature of disordered breathing such as sleep apnoea. The net result is instability in the respiratory pattern, with breath-to-breath intervals varying widely, from a more regular pattern prior to hypoxia. This is thought to be due to disturbances in central signalling, though there is some evidence that carotid body chemoafferent discharge is responsible.

In the analysis of regularity of physiological signalling, a method to quantify regularity has been the use of Poincaré plots. This plot is also known as a “return plot”, where the interval between breaths is plotted against the subsequent interval (Equation 2.4).

In a system devoid of variability, the plot will be a single geometric point, whereas in a chaotic system, or one beset by random noise, the points would

be widely scattered, with no pattern. The most common pattern in biological systems is an ellipse (Figure 2.17). Over long periods of time, it is reasonable to expect that SD2 would change for any parameter, e.g. respiratory rate increasing with exercise, but in this dimension the adjacent interbreath interval remains relatively constant. A large value of SD1 is however a metric of short term instability, that each interbreath interval is not correlated with the next. Breathing is irregular.

This has been applied to heart rate variability with great success, with characteristic patterns denoting risk of developing ventricular dysrhythmia after heart attack (Huikuri et al., 1996) and the automated diagnosis of atrial fibrillation (Park et al., 2009). Though less commonly applied to studying ventilation, strain differences in reoxygenation breathing have been described (Gonsenhauser et al., 2004). Peng et al. (2011) used this method of analysis to qualitatively describe the increased variability in air-breathing HIF-2 $\alpha$  mutants.

**Equation 2.4** Defining a Poincaré plot

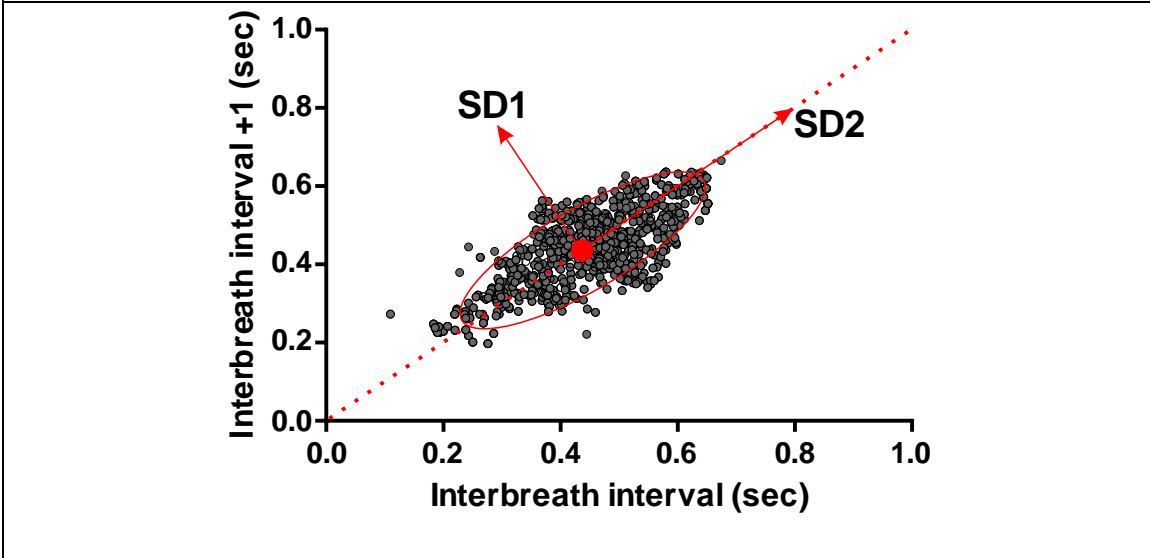
If the interval between breaths is denoted:

$$t_1, t_2, t_3, t_4, t_5, t_6, t_7, \dots$$

Then, a Poincaré plot will plot the points:

$$(t_1, t_2), (t_2, t_3), (t_3, t_4), (t_4, t_5), (t_5, t_6), (t_6, t_7), \dots$$

**Figure 2.17** Example of a Poincaré plot (graphically generated data). The typical pattern of a physiological breathing pattern is an ellipse. The centroid (the mean value in each axis, is the centre of the ellipse. The bounds of the ellipse along the line of identity and its perpendicular at the centroid can be quantified as two distinct standard deviations, SD1, and SD2.



## **Generation of genetically engineered mice**

### **Overview**

Gene targeting to inactivate single genes in mice has become a powerful technology since the technique was described in the late 1980s (Schwartzberg et al., 1989). By introducing a mutant sequence into the genome of mice through homologous recombination, normal gene function can be disrupted throughout all cells in the animal. This has the advantage of perturbing gene expression, without pharmacological intervention, with wildtype as control. It is recognised however, that by deleting a gene in the genome, the selection pressure for other phenotypic traits is enhanced. So, although the role of a gene can be studied through its absence, it cannot be excluded that other genes are being expressed abnormally in a knockout, confounding any observations. As yet, no conditional (where genes are knocked out only in a specific tissue) nor inducible knockouts (where the gene is perturbed in an animal that has developed with the gene present) of TASK 1 or TASK 3 have been created.

### **Generation of TASK 1<sup>-/-</sup> knockouts**

The founder TASK 1 knockout mice were generated by the Laboratory of Prof. Steven Brickley, Imperial College London, were a gift to Prof. Keith Buckler and were subsequently maintained at the Biomedical Science Building, University of Oxford.

Aller et al. (2005) described that TASK 1 was identified from a mouse BAC catalogue, and a vector containing Sph1 and EcoR1 was spliced into exon 1 of the first transmembrane (TM) domain. This vector allowed the insertion of a TAG3IRESlacZpAneopA cassette into the TASK 1 gene. During insertion of the

cassette, it was identified that rather than achieving an insertion downstream of the initiating codon of the first TM domain, the cassette was unintentionally inserted upstream of the initiating codon of TASK 1. In doing so, a deletion of 72bp occurred. Following electroporation into embryonic stem (ES) cells (mouse strain 129/sv) and homologous recombination, the internal ribosomal entry site (IRES) still supported lacZ translation to disrupt the gene. Targeted ES cells were identified and injected into mouse blastocysts, before maintaining their offspring on a C57BL6 inbred background strain.

The resulting genomic TASK 1 knockout mice were described as having generally normal behaviour, but poor motor performance on a rotarod and walking beam tests (Aller et al., 2005). Subsequent characterisations of these mice have described defects in aldosterone synthesis (Heitzmann et al., 2008), a decreased sensitivity to hypoxia (Trapp et al., 2008) and an increase in the volatile anaesthetics halothane and isoflurane required to achieve MAC endpoints (Linden et al., 2006). Independently created TASK 1 knockouts have shown impaired T-cell proliferation and cytokine production, suggesting a role in mediating CNS inflammation (Bittner et al., 2009), but normal central CO<sub>2</sub> chemosensitivity (Mulkey et al., 2007).

### **Generation of TASK 3<sup>-/-</sup> knockouts**

The founder TASK 3 knockout mice were generated by the Laboratory of Prof. William Wisden, University College London, and were also a gift to Prof. Keith Buckler and subsequently maintained at the Biomedical Science Building, University of Oxford.

In common with the TASK 1<sup>-/-</sup> animals described above, the TASK 3 gene was isolated from a mouse BAC catalogue. Here, the method used a modified pLitmus vector to target exon 1 of TASK 3 (Brickley et al., 2007). By using a loxP sequence in the targeting vector, it was intended that this locus would be flanked by loxP sequences (ie “floxed”), in order to create an inducible knockout. Unfortunately, when the TASK 3 mice after gene targeting were crossed with flp deleter mice to remove the neomycin resistance gene (which is only present to identify homologous recombination in ES cells), the loxP sites were inverted. So, although the targeting event was not successful in creating a floxed TASK 3 exon 1, the cassette itself proved to disrupt TASK 3 function, representing a genomic knockout. The authors addressed the concern that leaving a neomycin resistance sequence as part of the mutant gene affects expression adjacent genes, and concluded that in this case it would not. Subsequent phenotyping of these animals revealed nocturnal hyperactivity and deficits in proxy tests of cognitive function (Linden et al., 2007), now thought to be an analogous condition to the TASK 3 related Birk-Barel retardation in humans (Veale et al., 2014).

### **Backcross of single TASK knockout lines**

Previous reports of both TASK 1 and TASK 3 single channel knockout mice allude to their maintenance on a C57BL6 background. However, the degree to which they are congenic with this strain has not been reported. Without knowing the true background strain, it would take a full backcross cycle (approximately 18-24 months) to achieve an inbred background, where only the only loci that differ are those disrupted for experimental purposes. Differences in the ventilatory phenotype between inbred strains has been well reported

(Tankersley et al., 1994), and it is of significance that C57BL6 mice themselves have in the past been used as models of sleep-disordered breathing (Yamauchi et al., 2008; Yamauchi et al., 2010). Using litter-mate controls is one way of ameliorating this “background” effect, though it does not exclude the effect of adjacent genes having a significant phenotypic effect (Kumar et al., 2004; Lusic et al., 2007; Eisener-Dorman et al., 2009).

An alternative is to interbreed mutant animals with the background strain of choice. At 20 brother-sister filial generations of a “backcross”, a technique which predates knockout technology, it is theoretical that 98.7% of the genome will be homologous (Green, 1981). However, this relies on the genome being infinitely divisible, and truly random assortment occurring at each generation. The advantage of creating a congenic animal is that the inbred strain can act as a control dramatically reducing animal use, as the colony can be maintained as homozygotes. This is especially important with an animal with two mutant alleles, where the double knockout progeny of double heterozygote parents is 1:16.

To reconcile these two limitations, we used a panel of 384 single nucleotide polymorphisms (SNP) to characterise the background strain of the mutant animals (Marker assisted accelerated backcrossing, Charles River, Margate, Kent, UK). Following three generations of brother-sister matings, we achieved >90% homology with C57BL6 in both TASK knockout strains (Table 2.4).

**Table 2.4** Measuring the homology of TASK knockout animals with inbred C57BL6 animals, before and after backcross

Strain	Initial homology (%)	Resultant homology (%)
TASK 1 <sup>-/-</sup>	80.03	94.62
TASK 3 <sup>-/-</sup>	76.31	91.99

### Generation of TASK 1<sup>-/-</sup>/3<sup>-/-</sup> knockout

Following the backcross of each line described above, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals were interbred. It took four months to produce each founder breeder from double heterozygote parents (TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup>). With an average litter size of eight, Mendelian inheritance would suggest that double the observed number of progeny from TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> breeders would be double knockouts. However, once created, double knockouts interbred with eventually normal fertility and litter sizes. It is possible that this represents an in utero selection pressure against double knockouts.

### Genotyping of knockouts

Tissue from ear biopsies was used as template genomic DNA, isolated using the DNEasy system (Qiagen, Manchester, UK).

Both TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup> and TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> can be genotyped using polymerase chain reaction and gel electrophoresis. For TASK 1<sup>-/-</sup>, this involves amplification of the lacZ insertion used to create the mutant gene (Table 2.6), and concurrently, but in a different reaction, to amplify native TASK 1 (Table 2.5) to ensure that the animal is not heterozygous. For TASK 3<sup>-/-</sup>, only one reaction is required (Table 2.7), as due to the nature of the knockout a larger gene product is produced when a mutant allele is present. Therefore, two bands

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represent a heterozygous animal, and single bands of differing weights (Figure 2.18) represent wildtype (light band) and knockout (heavy band).

When the double knockout was being recapitulated, the process of three polymerase chain reactions and three agarose gels became time inefficient and relatively costly, given the low yield of homozygote offspring. To streamline this process, in conjunction with Transnetyx (Cordova, United States), a qPCR based assay was used.

Instead of relying on the molecular weight of PCR amplified products, qPCR uses sequence-specific primers, with an integrated fluorophore and fluorescence quenching motif. Polymerase enzyme liberates the fluorophore which is detected as fluorescence. The exponential increase in reporter fluorescence is characteristic of target sequence presence (Figure 2.19).

**Table 2.5** Procedure for identifying TASK 1 gene by polymerase chain reaction

Primers (5' to 3')	Forward CGGCGTGGAGATCCGCGAG Reverse ACACGGAGGAGTGGCGCTCT	
Thermocycle sequence	<div style="text-align: center;">                     15 minutes @ 95°C                       1 minute @ 94°C                      1 minute @ 61.9°C                      1 minute @ 72°C                 </div>	<div style="text-align: center;">                     }                      Repeat                      36 times                 </div>
Expected product	Wildtype: Knockout Heterozygote	759bp product No product 759bp product

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**Table 2.6** Procedure for identifying inserted lacZ sequence as an indicator of the presence of a mutant TASK 1 gene

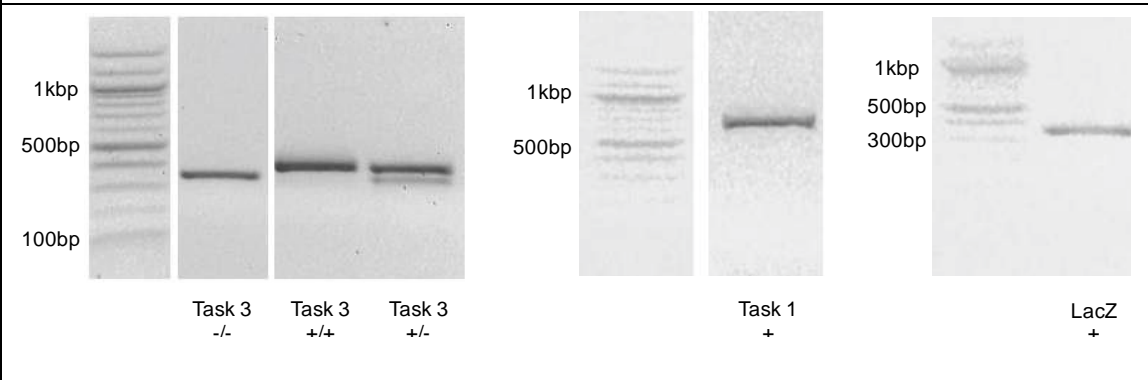
Primers (5' to 3')	Forward TTCACTGGCCGTCGTTTTACAACGTCG Reverse TGAGCGAGTAACAACCCGTCGGATTCT	
	15 minutes @ 95°C	
Thermocycle sequence	1 minute @ 94°C 1 minute @ 61.5°C 1 minute @ 72°C	} Repeat 36 times
	10 minutes @ 72°C	
Expected product	Wildtype: No product Knockout 364bp product Heterozygote 364bp product	

**Table 2.7** Procedure for identifying TASK 3 wildtype and mutant genes by polymerase chain reaction

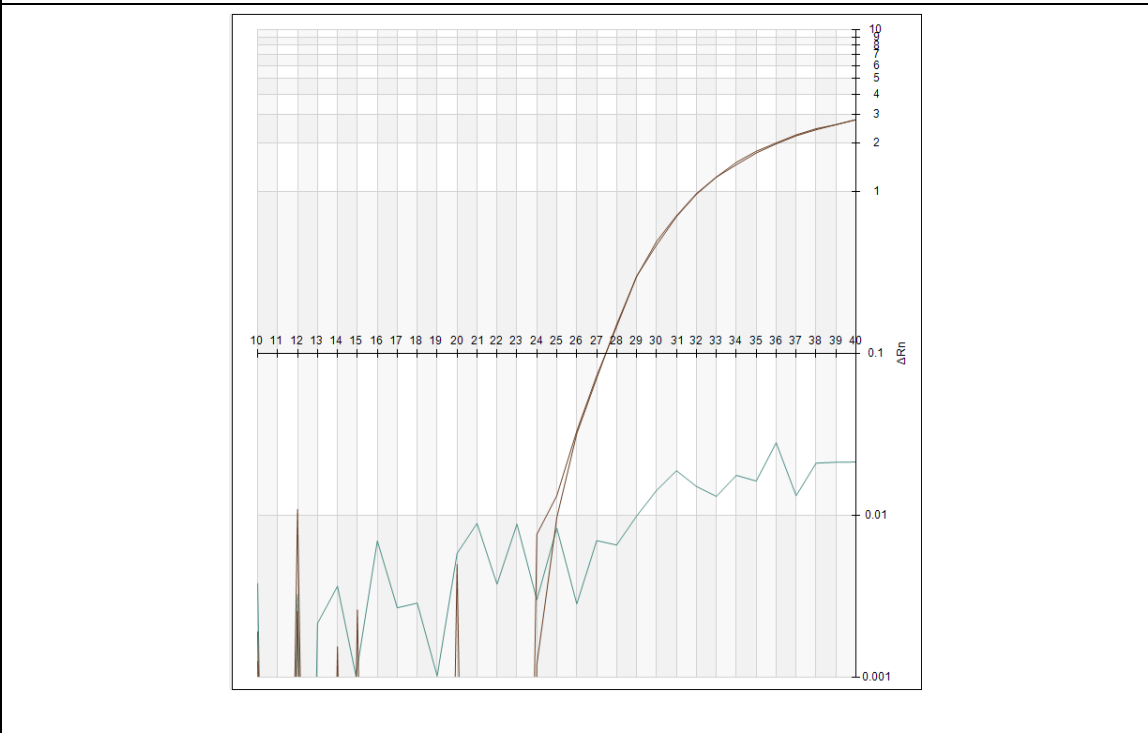
Primers (5' to 3')	Forward CCGGGTCTCCCTCTGTCCCG Reverse GGAGCACCCAGTGCAACGGA	
	15 minutes @ 95°C	
Thermocycle sequence	1 minute @ 94°C 1 minute @ 60.5°C 1 minute @ 72°C	} Repeat 33 times
	10 minutes @ 72°C	
Expected product	Wildtype: 301bp product Knockout 351bp products Heterozygote 301 and 351bp products	

**Figure 2.18** Example gel of PCR products of genotyping three animals for the TASK 3 gene. Also shown are representative gene products for TASK 1 and LacZ. To fully genotype for TASK 1, both alleles need to be amplified. In this example, the genotype would be heterozygous for TASK 1. The reference ladder is as marked, lighter bands are 100bp, heavier at 500bp intervals.

*Note that these gels have been cropped and cut for illustration purposes only, and the breaks are made clear.*



**Figure 2.19** Example of a qPCR amplification trace. Here, the mutant TASK 3 gene is amplified at 27 cycles, whereas the wild-type sample (blue) does not amplify by termination at cycle 40. Image courtesy of Transnetyx.



**Statistical methods**

Data are shown as means  $\pm$  SEM, unless otherwise stated. In general, the process of comparison was first by way of factorial ANOVA (the factors and levels described in each chapter). Only if ANOVA (and relative interactive terms) yielded significant results were post-hoc tests conducted with relevant corrections for multiple comparisons. Where just two groups were compared Student's t-test or non-parametric tests will be used as appropriate.

**Chapter 3:**

**VENTILATORY PHENOTYPES OF TASK CHANNEL**  
**KNOCKOUT MICE AND INTERACTIONS WITH VOLATILE**  
**ANAESTHETICS**

## **Overview**

The volatile anaesthetic agents depress minute ventilation in a dose dependant fashion. At subanaesthetic doses (<0.2 MAC) quiet breathing is not affected, but there is a profound and specific depression of AHVR in both humans (Knill and Gelb, 1978; van den Elsen et al., 1995; Pandit et al., 1999a) and other animals (Ponte and Sadler, 1989b; Karanovic et al., 2010). This depression is clinically relevant, as subanaesthetic doses of volatile agents are known to persist for many hours after administration has finished (Yasuda et al., 1991; Lockwood, 2010) when patients are particularly likely to experience hypoxaemia (Moller et al., 1990; Pedersen et al., 2009; Sun et al., 2015).

The magnitude of this depression seems agent-specific in humans (Pandit, 2002), with relative potency of halothane > enflurane > isoflurane > sevoflurane. This order of potency appears conserved across organisational hierarchies and across species. The same order is, for example, observed in rats (Karanovic et al., 2010). Furthermore, the same order of potency was reported (for halothane vs sevoflurane) in the hypoxia-induced glomus cell  $[Ca^{2+}]_i$  response (Pandit and Buckler, 2009), and  $K_B$  (native TASK) channel activity (for halothane vs isoflurane) in euoxia and hypoxia (Pandit et al., 2010b).

Although there are several candidate mechanisms with the carotid body proposed as mediators of  $O_2$  sensing (Kumar and Prabhakar, 2011),  $K_B$  channels are of particular interest as, uniquely as compared with other candidate systems, they are sensitive to both volatile anaesthetic agents and hypoxia (Buckler et al., 2000). They therefore offer a parsimonious explanation for the anaesthetic depressive effects noted above.

### *Chapter 3 - Ventilatory phenotypes of TASK channel knockout mice and interactions with volatile anaesthetics*

These channels are part of the  $K_{2P}$  family, which are expressed not only in the carotid body, but are widespread throughout the central and peripheral nervous system (Medhurst et al., 2001). They exist as homomers (TASK 1 or TASK 3) or dimerise into TASK 1/TASK 3 assemblies (TASK 1/3) (Kim et al., 2009), and are respectively products of the *Kcnk3* and *Kcnk9* genes.  $K_B$  channels generate a leak current, which influences resting membrane potential in excitable cells. Hypoxia inhibits  $K_B$  opening (decreases open probability), leading to membrane depolarisation. Halothane and isoflurane both activate  $K_B$  channels in glomus cells (Pandit et al., 2010b).

Preliminary experiments in expressed TASK channels appear to show similar differential effects between halothane and isoflurane (Berg et al., 2004) as previously reported in humans (Pandit, 2002), rats (Karanovic et al., 2010) and glomus cells (Pandit and Buckler, 2009; Pandit et al., 2010b). HEK cells expressing only TASK 1 exhibited an increase in potassium conductance (channel opening) only with halothane but not isoflurane while TASK 1/3 heterodimers and TASK 3 homomers showed increased conductance in both isoflurane and halothane. As such, the TASK 1 subunit of the heterodimeric  $K_B$  channel may be regarded as relatively isoflurane “insensitive” but, like the TASK 3 subunit, halothane sensitive.

Studies of mice carrying mutations or deletions of neurotransmitter or ion channel genes present a powerful approach to understanding the in vivo mechanism of anaesthetic action.

Previous studies have addressed hypoxic and anaesthetic hypnotic sensitivity separately in TASK knockout mice, but not the specific effects that volatile

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agents have when these interventions are combined, i.e., the anaesthetic influence on AHVR (Linden et al., 2006; Linden et al., 2007). Linden et al. (2006) reported decreased sensitivity to the hypnotic effects (loss of tail withdrawal reflex) of halothane and isoflurane for single knockouts of both TASK 1 and TASK 3 (Linden et al., 2007). However, changes in anaesthetic sensitivity did not strictly correspond to changes in AHVR. In the absence of anaesthetic, murine knockouts for TASK 3 (i.e., TASK 1 persisting) exhibited a normal AHVR whereas knockouts for TASK 1 (i.e., TASK 3 persisting) exhibited a reduced AHVR (Trapp et al., 2008). In the light of these findings, it was unusual that murine double knockouts (i.e., TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup>, TASK 1 and 3 absent) were found to have a normal AHVR (Mulkey et al., 2007), though no data were presented for this finding. Importantly, these studies were conducted with poikilocapnic hypoxia, which is known to underestimate an hypoxic response (Steinback and Poulin, 2007). Whether there is an intermediate phenotype in heterozygous animals has not been studied either. In other ventilation studies in mice, it has been demonstrated that heterozygous animals have a distinct phenotype when compared to both knockouts and controls (Peng et al., 2011; Bishop et al., 2013). This is of particular use when a knockout phenotype is severe.

In all genetic models, there is the potential that a mutation intended to ablate a gene results in an aberrant protein that functions in an unanticipated manner. This is particularly the case in the TASK 3<sup>-/-</sup>, due to the persistence of the engineered neomycin resistance gene (Brickley et al., 2007), which can influence transcription of other genes (Pham et al., 1996). TASK 1 mutations

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have also been demonstrated to have a dominant negative influence on TASK 3 channel activity (Berg et al., 2004). If this were the case the TASK 3 function could be influenced in TASK 1<sup>-/-</sup> animals despite not being targeted for ablation. For this reason, studying the double heterozygote (TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup>) is informative. A normal heteromultimer could still form in this genotype, possibly resulting in a normal ventilatory phenotype. On the other hand, a finding of a deficit in TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> AHVR, would suggest a more complex interdependence between TASK 1 and TASK 3, either through gene dosing, or dominant negative mutations.

If both AHVR and the sensitivity to anaesthetic are, at least in part, both mediated by family of TASK channels, and further, if the depression of AHVR by anaesthetic agents is mediated by a highly conserved mechanism across species, then it would be expected that the order of potency as established in humans (Pandit, 2002) would be similarly reflected in wildtype mice.

Furthermore, from the preliminary results reported by others in expressed channel systems as discussed above, it can be hypothesised that:

- (1) Knocking out the TASK 3 gene would manifest as a (a) normal AHVR in absence of anaesthetic, (b) a relative resistance to depression of AHVR by isoflurane but (c) more profound depression of AHVR by halothane.
- (2) In contrast, knocking out the TASK 1 gene would result in (a) a reduced AHVR in absence of anaesthetic, (b) retained sensitivity to depression of AHVR by isoflurane and (c) retained sensitivity to depression of AHVR by halothane.

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- (3) TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> would exhibit (a) a depressed AHVR in the absence of anaesthetics; (b) a retained AHVR in the presence of both halothane and isoflurane.
- (4) TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> would have an intermediate AHVR when compared to TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> and C57BL6.

The aims of this part of my thesis were as follows:

- (1) To characterise the ventilatory responses of wildtype (C57BL6) and TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup>, ('single KOs') TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> ('double KO') and TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> ('double HET') mice to hypoxia and hypercapnia to assess if these were concordant with previous reports.
- (2) To assess if the AHVRs of wildtype, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> mice are differently sensitive to halothane and isoflurane, as predicted (i.e., with the latter being relatively insensitive to effects of isoflurane).
- (3) To assess the effects of halothane and isoflurane on ventilatory response to CO<sub>2</sub> in TASK1<sup>-/-</sup> and TASK3<sup>-/-</sup> mice (an as yet unexplored phenomenon in the literature).

## **Methods**

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986. The animals were of C57BL6 background and colonies were maintained in a standard animal facility in groups of 1-8, in individually ventilated cages. Lights were on from 0600 to 1800 with food pellets and water available *ad libitum*. C57BL6 wildtype mice were supplied by Harlan. All experiments were conducted in the 7-9<sup>th</sup> week of life on male animals only.

### *Chapter 3 - Ventilatory phenotypes of TASK channel knockout mice and interactions with volatile anaesthetics*

Whole body plethysmography was performed as described in Chapter 2 – General Methods. Oxygen, carbon dioxide and volatile anaesthetic concentration within the chamber were measured by continuous sampling from a side-port (Datex Capnomac Ultima). Animals were acclimatised to the chambers for a period of 30 minutes prior to experimentation. All experiments were conducted at room temperature, between 0800 and 1400.

Hypoxic stimulus was performed with 5-minute step changes from air into 10% O<sub>2</sub>, 90% N<sub>2</sub> (poikilocapnic hypoxia), 10% O<sub>2</sub>, 3% CO<sub>2</sub>, 87% N<sub>2</sub> (isocapnic hypoxia). Response to carbon dioxide was assessed with 5-minute step changes from air into 1% CO<sub>2</sub>, 3% CO<sub>2</sub>, 4% CO<sub>2</sub> and 5% CO<sub>2</sub>, each in balanced oxygen as well as euoxic hypercapnia (21% O<sub>2</sub>, 4% CO<sub>2</sub>, 75% N<sub>2</sub>). Ventilatory responses in the presence or absence of anaesthetic was measured using isocapnic hypoxia and euoxic hypercapnia. A sham switch was carried out for each genotype using the same experimental setup, but switching from air to air, to assess if disturbances in air flow might influence ventilation.

Gases were delivered from pressurised cylinders, supplied and calibrated by BOC (Guildford, UK). Volatile anaesthetics were vaporised in plenum-style vaporisers delivered by individual rotameters to each chamber at flows of 2 l.min<sup>-1</sup>. The order of gas inputs was randomised and animals undertook experiments no more than 3 times over 5 days.

#### **Experimental protocols**

30 minutes of acclimatisation to the chambers followed by one of;

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1. Poikilocapnic hypoxia: 5 minutes of air breathing, then 5 min hypoxia 10% followed by return to air-breathing;
2. Isocapnic hypoxia: 5 min air breathing, followed by 5 min hypoxia, with 3% added CO<sub>2</sub>, then return to air breathing;
3. Euoxic hypercapnia: 5 min air breathing, followed by 5 min breathing 21% O<sub>2</sub> 4% CO<sub>2</sub>, then return to air breathing.

Protocols 2 and 3 were repeated with background 0.1 to 0.7 minimal alveolar concentration (MAC) anaesthetic at concentrations 0 to 1% (the MAC of mice was taken with respect to loss of tail withdrawal reflex and adjusted for genotype (Table 3.1).

Genotypes were confirmed in the knockout strains by the methods described in Chapter 2. Halothane was supplied by Meriel (Harlow, UK) and isoflurane by Abbott (Abindgon, UK).

**Table 3.1** Agent specific anaesthetic MAC values adjusted for genotype (Sonner et al., 2000; Linden et al., 2006; Linden et al., 2007).

<b>Genotype</b>	<b>MAC halothane (%)</b>	<b>MAC isoflurane (%)</b>
C57BL6	1.25	1.38
TASK 1 <sup>-/-</sup>	1.32	1.36
TASK 3 <sup>-/-</sup>	1.48	1.5

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Ventilatory (acute hypoxic and hypercapnic) responses were subjected first to factorial analysis of variance, with the factors being ‘mouse’ (five levels, one for each genotype: wildtype, TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup>, TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup>, TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup>), ‘drug’ (three levels, none, halothane, and isoflurane), ‘dose’ (four levels, one for each concentration used), ‘O<sub>2</sub>’ (two levels, 10% and 21% O<sub>2</sub>) and ‘CO<sub>2</sub>’ (six levels, from 0% to 5% CO<sub>2</sub>). The model and interactive terms were in turn chosen to address the specific hypothesis. Where significance was suggested, post-hoc Bonferroni tests were applied. Results are presented as mean ± SEM unless otherwise stated.

## **Results**

### **Baseline variables during euoxic ventilation**

Wildtype mice weighed less than their age- and gender-matched single channel knockouts but there was no difference between the weights of the knockouts (Table 3.2, ANOVA p=0.562, p=1.0).

**Table 3.2** Animal weight on the day of experiment. ANOVA p<0.001. Post-hoc C57BL6 vs TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> p<0.001, all other comparisons not significant.

<b>Genotype (n)</b>	<b>Weight (g)</b>
C57BL6 (152)	21.8 ± 0.2
TASK 1 <sup>-/-</sup> (36)	26.1 ± 0.3
TASK 3 <sup>-/-</sup> (56)	27.1 ± 0.3
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup> (43)	22.8 ± 0.6
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup> (8)	23.5 ± 1.6

### *Chapter 3 - Ventilatory phenotypes of TASK channel knockout mice and interactions with volatile anaesthetics*

Genotype had a modest, but statistically significant effect on euoxic minute ventilation (Table 3.3, ANOVA  $p=0.028$ ). At post-hoc analysis, this genotype effect was due to the significant difference between the minute ventilation of C57BL6 vs TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> ( $3.3 \pm 0.1$  vs  $3.1 \pm 0.1$  ml·g<sup>-1</sup>·min<sup>-1</sup>  $p=0.042$ ). The effect of genotype on minute ventilation was reflected in a significant genotype influence on both respiratory rate ( $p<0.001$ ) and tidal volume ( $p<0.001$ ). Post-hoc analysis revealed C57BL6 had significantly smaller tidal volume than TASK 1<sup>-/-</sup> ( $p=0.022$ ) and double HET ( $p<0.001$ ). Overall the double HET had a greater tidal volume than any other genotype ( $p<0.001$  for all) except TASK 1<sup>-/-</sup> where the interaction was not significant ( $p=0.1$ ).

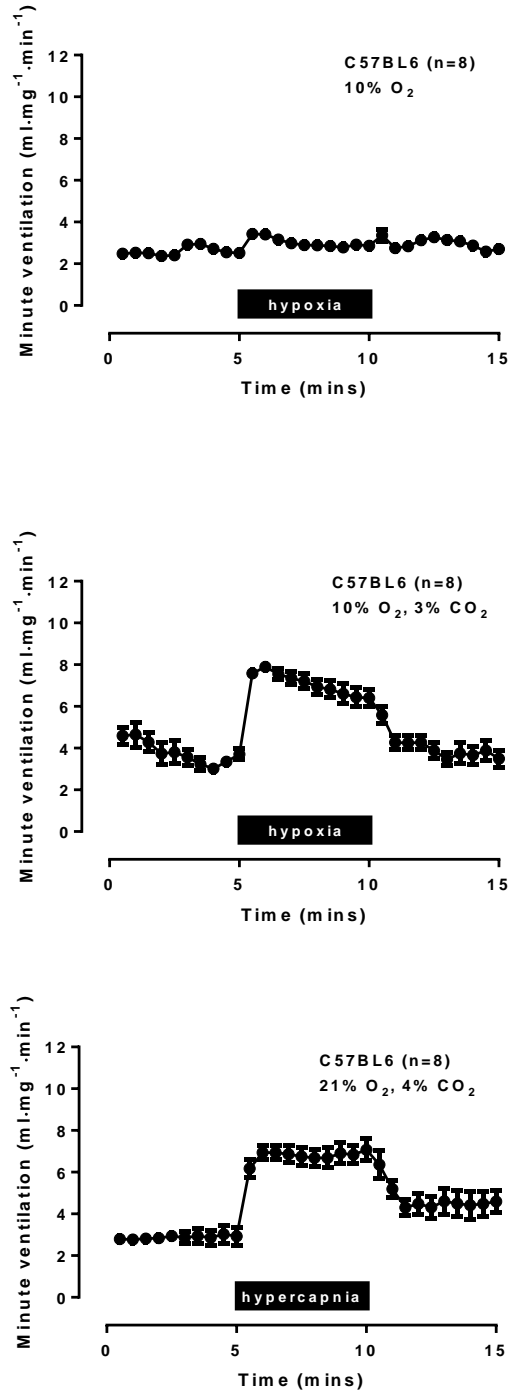
C57BL6 had significantly lower respiratory rates than TASK 1<sup>-/-</sup> ( $p<0.001$ ) and TASK 3<sup>-/-</sup> ( $p=0.003$ ).

There was no significant change in ventilation for any genotype with sham switching of inspired gas ( $p=0.76$ ).

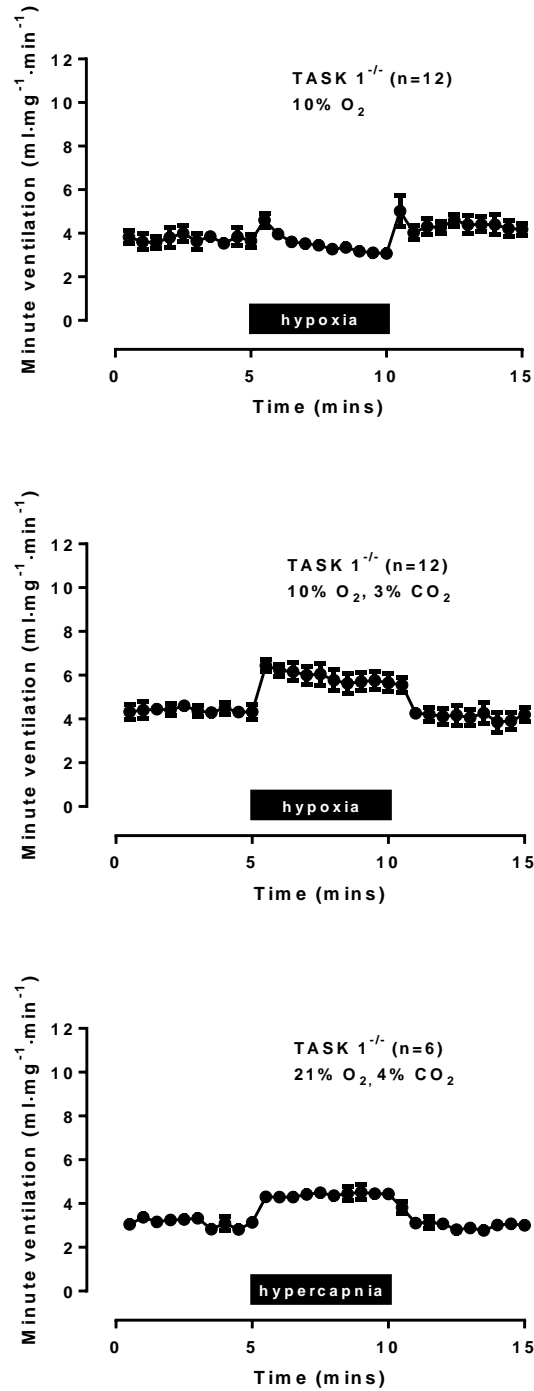
#### **Ventilatory responses to hypoxia: isocapnic vs poikilocapnic responses**

Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4 and Figure 3.5 show minute ventilation traces for hypoxia and hypercapnia across all the genotypes studied. Data are shown for hypoxic exposures in poikilocapnia, background 3% CO<sub>2</sub> (isocapnia) and 4% CO<sub>2</sub> (hypercapnia). The 3% CO<sub>2</sub> data were used to calculate the isocapnic AHVR values. The 4% CO<sub>2</sub> data were used, with 3% CO<sub>2</sub> data, to construct ventilation-CO<sub>2</sub> relationships with and without hypoxia (see Figure 3.8).

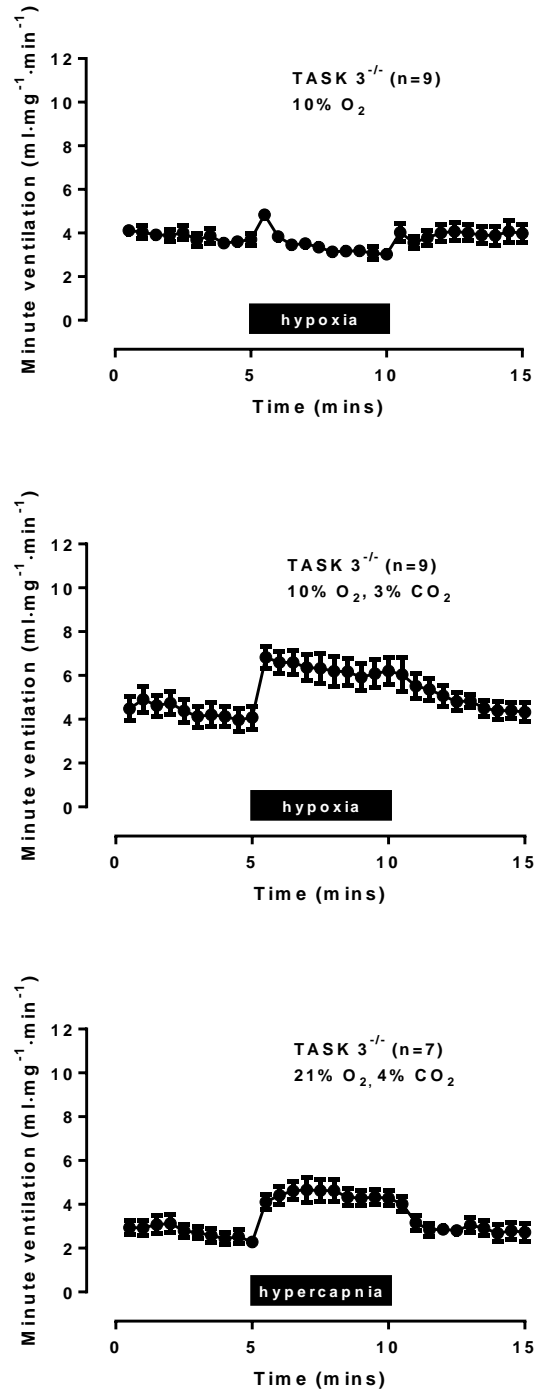
**Figure 3.1** Wildtype ventilatory responses to poikilocapnic and isocapnic hypoxia, and hypercapnia.



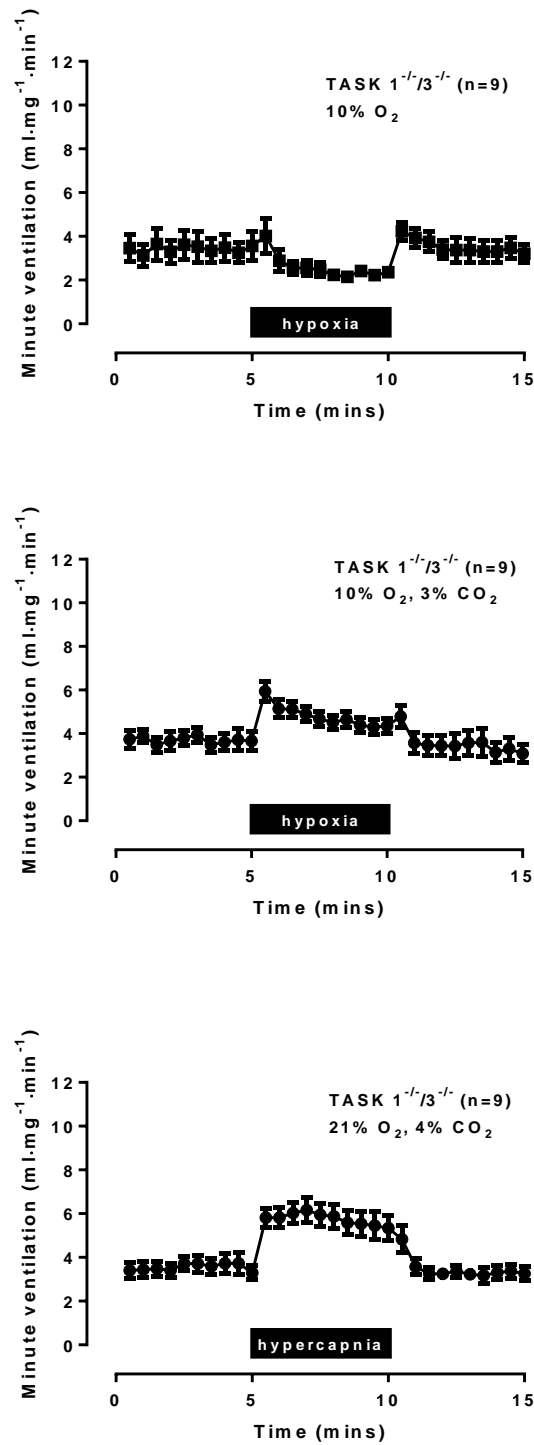
**Figure 3.2** TASK 1<sup>-/-</sup> responses to poikilocapnic and isocapnic hypoxia, and hypercapnia.



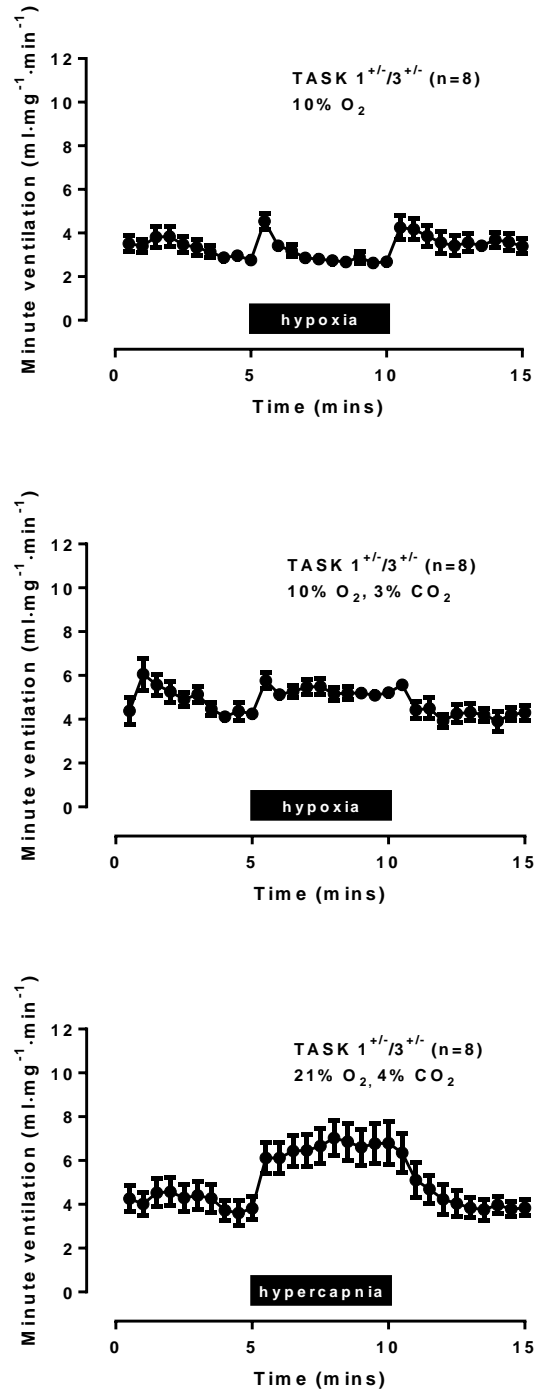
**Figure 3.3** TASK 3<sup>-/-</sup> responses to poikilocapnic and isocapnic hypoxia, and hypercapnia.



**Figure 3.4** TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> responses to poikilocapnic and isocapnic hypoxia, and hypercapnia.



**Figure 3.5** TASK  $1^{+/-}/3^{+/-}$  responses to poikilocapnic and isocapnic hypoxia, and hypercapnia.



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Neither single knockout had a significant change in minute ventilation during poikilocapnic hypoxia (TASK 1<sup>-/-</sup>, p=0.747 and TASK 3<sup>-/-</sup>, p=0.976) (Figure 3.2, Figure 3.3, Table 3.4 and Table 3.5).

However, there was a significant decrease in ventilation in the double KOs (mean 3.42 to 2.67 ml·g<sup>-1</sup>·min<sup>-1</sup>, p=0.033) early during poikilocapnic hypoxia (Figure 3.4). The only significant overall influence of genotype on the poikilocapnic response was between C57BL6 and double KOs (*post-hoc*, p=0.001), the largest positive and negative responses. The small, but significant increase in ventilation in the C57BL6 animals was reduced by 50% by the end of the hypoxic exposure (Table 3.4).

In contrast, isocapnic hypoxia caused an increase in minute ventilation across all genotypes (Figure 3.1, Figure 3.2, Figure 3.3, Figure 3.4, Figure 3.5 and Table 3.6). The magnitude of this response was significantly greater for C57BL6 than TASK 1<sup>-/-</sup> (p<0.001), TASK 3<sup>-/-</sup> (p=0.002), TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> (p<0.001) and TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> (p<0.001). A characteristic decline (HVD) was seen in all genotypes (Table 3.6).

There were no significant differences between the isocapnic AHVR of TASK mutant animals. The difference between C57BL6 and the TASK knockout mice was due to significantly different change in tidal volume (p<0.001) and respiratory rate (p<0.001) (Figure 3.6).

**Table 3.3** Euoxic values for minute ventilation, respiratory rate and tidal volume across genotypes. The data are presented as mean  $\pm$  SEM.

Genotype(n)	Minute ventilation in air ( $\text{ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ )	Respiratory rate ( $\text{breaths}\cdot\text{min}^{-1}$ )	Tidal volume ( $\text{ml}\cdot\text{g}^{-1}$ )
C57BL6 (120)	$3.3 \pm 0.1$	$211 \pm 10$	$12.6 \pm 0.2$
TASK 1 <sup>-/-</sup> (56)	$3.2 \pm 0.1$	$283 \pm 16$	$14.6 \pm 0.6$
TASK 3 <sup>-/-</sup> (66)	$3.1 \pm 0.1$	$288 \pm 15$	$12.7 \pm 0.5$
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup> (125)	$3.1 \pm 0.1$	$256 \pm 8$	$11.8 \pm 0.2$
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup> (24)	$3.6 \pm 0.2$	$306 \pm 14$	$15.6 \pm 1.3$

**Table 3.4** Summary of minute ventilation in euoxia and the peak and end poikilocapnic hypoxia. There is a characteristic decline in minute ventilation during hypoxia (HVD), with only C57BL6 animals maintaining ventilation above euoxic values after five minutes exposure to 10% O<sub>2</sub>. All values in  $\text{ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$  unless stated.

Genotype (n)	Euoxic minute ventilation	Peak hypoxic minute ventilation	End hypoxic minute ventilation	Hypoxic ventilatory decline (%)
C57BL6 (8)	$2.6 \pm 0.1$	$3.2 \pm 0.1$	$2.9 \pm 0.1$	50
TASK 1 <sup>-/-</sup> (6)	$3.8 \pm 0.2$	$3.7 \pm 0.1$	$3.1 \pm 0.2$	n/a
TASK 3 <sup>-/-</sup> (9)	$3.6 \pm 0.2$	$3.6 \pm 0.1$	$3.1 \pm 0.3$	n/a
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup> (6)	$3.4 \pm 0.6$	$2.7 \pm 0.4$	$2.3 \pm 0.2$	n/a
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup> (8)	$2.9 \pm 0.2$	$3.2 \pm 0.2$	$2.7 \pm 0.2$	60

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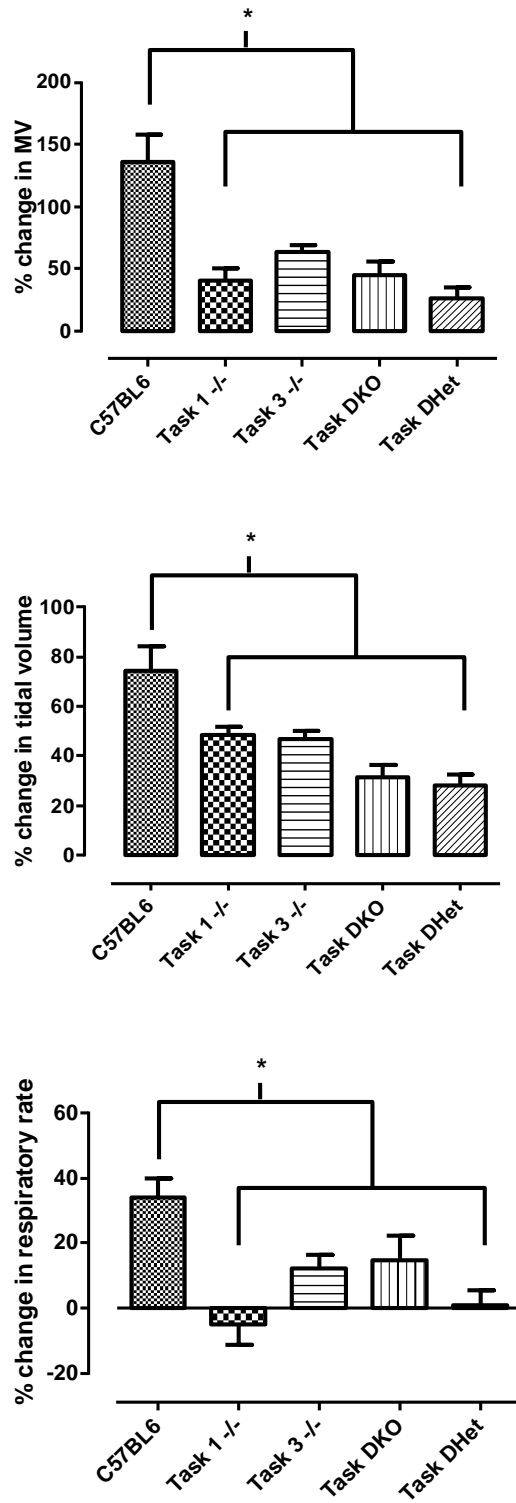
**Table 3.5** Ventilatory response to acute poikilocapnic hypoxia (10% O<sub>2</sub> in balance N<sub>2</sub>) across genotypes. Data are % ± SEM.

Genotype (n)	Acute poikilocapnic hypoxic ventilatory response (%)	Acute isocapnic hypoxic ventilatory response (%)
C57BL6 (8)	23.4 ± 7	136 ± 22
TASK 1 <sup>-/-</sup> (6)	-0.45 ± 4	40.9 ± 10
TASK 3 <sup>-/-</sup> (9)	1.1 ± 4	63.2 ± 7
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup> (6)	-16.8 ± 7	45.3 ± 11
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup> (8)	12.5 ± 9	27.0 ± 8

**Table 3.6** Summary of minute ventilation in euoxia and the peak and end isocapnic hypoxia. Again, a characteristic decline is observed during the exposure to hypoxia. All values in ml·g<sup>-1</sup>·min<sup>-1</sup> unless stated.

Genotype (n)	Euoxic minute ventilation	Peak hypoxic minute ventilation	End hypoxic minute ventilation	Hypoxic ventilatory decline (%)
C57BL6 (8)	3.4 ± 0.2	7.7 ± 0.3	6.5 ± 0.4	28
TASK 1 <sup>-/-</sup> (6)	4.5 ± 0.2	6.3 ± 0.4	5.7 ± 0.4	33
TASK 3 <sup>-/-</sup> (9)	3.8 ± 0.4	6.1 ± 0.4	5.8 ± 0.5	13
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup> (6)	3.7 ± 0.4	5.0 ± 0.4	4.3 ± 0.3	54
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup> (8)	4.2 ± 0.3	5.3 ± 0.2	5.2 ± 0.2	9

**Figure 3.6** Ventilatory responses to acute isocapnic hypoxia across genotypes. Data are %  $\pm$  SEM. \* denotes significance at  $p < 0.05$ .



### Ventilatory responses to hypercapnia

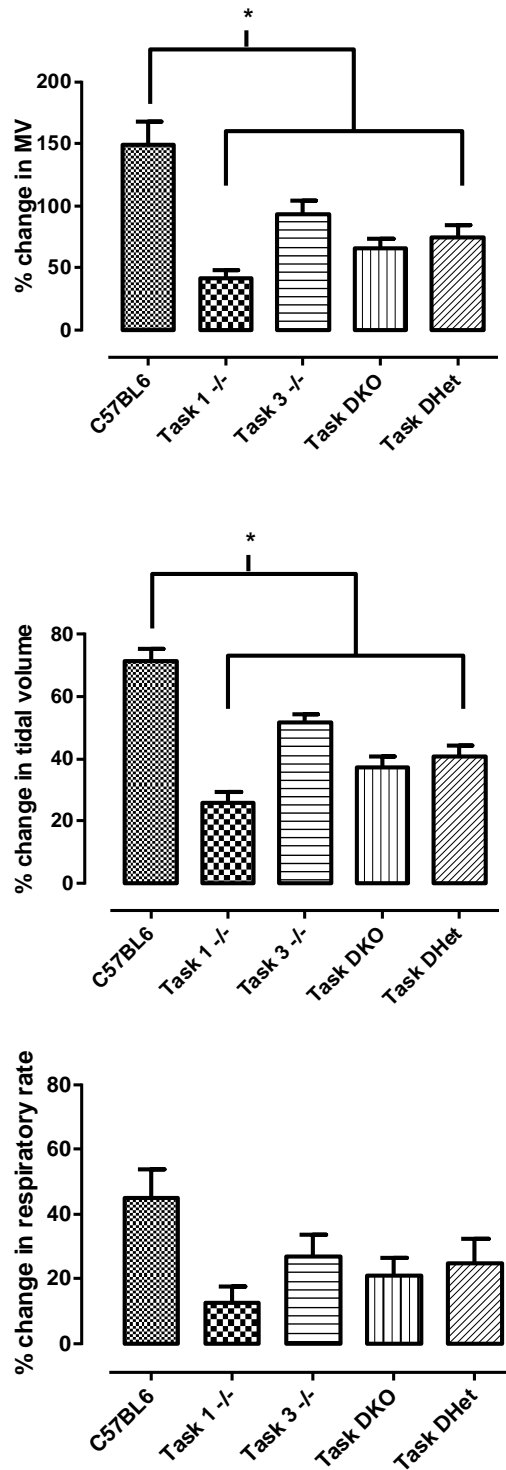
Addition of 4% CO<sub>2</sub> to the inspirate (hypercapnia) increased ventilation in all genotypes ( $p < 0.001$ , Table 3.7), most marked in the wildtype, then in TASK 3<sup>-/-</sup> and least in TASK 1<sup>-/-</sup>. C57BL6 had a significantly greater response to hypercapnia than TASK 1<sup>-/-</sup> ( $p < 0.001$ ), TASK 3<sup>-/-</sup> ( $p = 0.026$ ), TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> ( $p < 0.001$ ) and TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> ( $p = 0.001$ ). There were no significant differences between knockout strains.

**Table 3.7** Acute hypercapnic ventilatory response across genotypes. %  $\pm$  SEM.

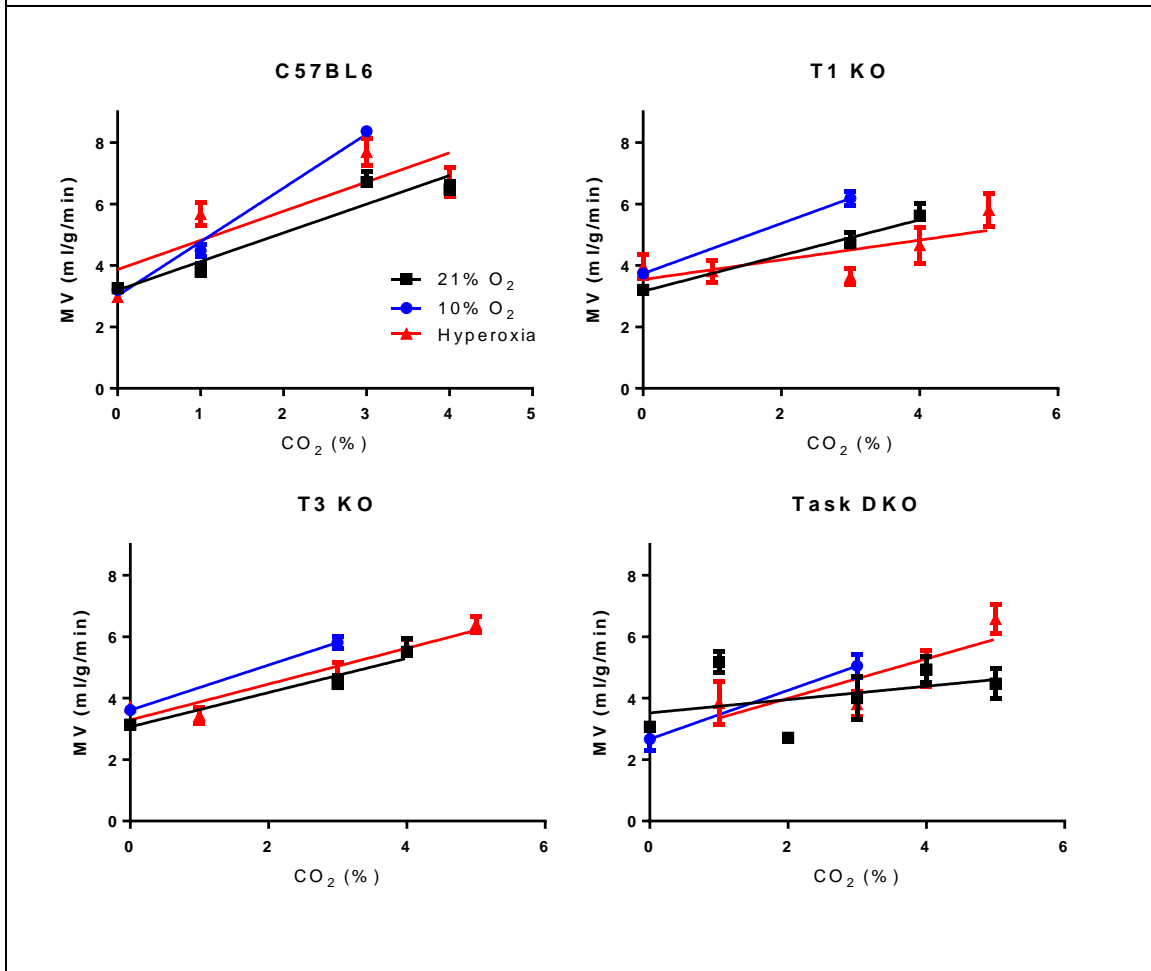
Genotype	Acute hypercapnic ventilatory response (%)
C57BL6	149 $\pm$ 19
TASK 1 <sup>-/-</sup>	41.7 $\pm$ 17
TASK 3 <sup>-/-</sup>	93.0 $\pm$ 11
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup>	65.4 $\pm$ 8
TASK 1 <sup>+/-</sup> /TASK 3 <sup>+/-</sup>	75.0 $\pm$ 10

There was a significant influence of genotype on the change in tidal volume ( $p < 0.001$ ) but not respiratory rate ( $p = 0.055$ ) in response to hypercapnia (Figure 3.7).

**Figure 3.7** Ventilatory responses to acute hypercapnia across genotypes. Data are %  $\pm$  SEM. \* denotes significance at  $p < 0.05$ .



**Figure 3.8** The difference between the gradient of CO<sub>2</sub> sensitivity in hypoxia and hyperoxia represents the contribution of the peripheral chemoreceptor to ventilation. Hyperoxia is >79% oxygen in all cases (dual gas mixtures of O<sub>2</sub> and CO<sub>2</sub>).



### Interactions of hypoxia and CO<sub>2</sub> with genotype

Minute ventilation was significantly influenced by both the level of O<sub>2</sub> ( $p < 0.001$ ) and CO<sub>2</sub> ( $p < 0.001$ ). This is represented by the differences in slopes in Figure 3.8. Genotype also had a significant influence on breathing ( $p < 0.001$ ), with C57BL6 always achieving a greater minute ventilation than each mutant strain (post-hoc, all interactions  $p < 0.001$ ). There was a highly significant interactive term combining hypoxia, CO<sub>2</sub> and genotype ( $p < 0.001$ ) for minute ventilation.

### Peripheral chemoreceptor response to hypoxia

Figure 3.8 shows the ventilatory responses to CO<sub>2</sub> in hyperoxia, normoxia and in hypoxia. The difference in gradient in hyperoxia represents the central response to hypercapnia, and is greatest in the C57BL6 genotype. The subtraction of the hyperoxia gradient from that in hypoxia represents the peripheral chemoreceptor response (to hypoxia). Qualitatively, the C57BL6 relationship is steeper than all other genotypes (Figure 3.8).

**Table 3.8** Gradients (ml/g/min per % CO<sub>2</sub>) of MV-CO<sub>2</sub> relationship, derived from the regression lines presented in **Figure 3.8**.

Genotype	Slope gradient (normoxia)	Slope gradient (hyperoxia)	Slope gradient (hypoxia)	Slope subtraction (hypoxia minus hyperoxia)
C57BL6	0.95	0.95	1.75	0.8
TASK 1 <sup>-/-</sup>	0.58	0.32	0.82	0.5
TASK 3 <sup>-/-</sup>	0.56	0.58	0.73	0.15
TASK 1 <sup>-/-</sup> /TASK 3 <sup>-/-</sup>	0.22	0.64	0.79	0.15

### Influence of anaesthetics on AHVR

Figure 3.9 shows the anaesthetic influence on hypoxic response across the wildtype and single knockout genotypes. Halothane at low doses quite clearly is universally depressant of the AHVR. Also shown is the very depressive action of halothane on euoxic ventilation especially in TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> and on the response to hypoxia across all genotypes. Isoflurane is also depressive on euoxic ventilation in the knockout strains, and the response to hypoxia across all genotypes, but appears less depressive than halothane.

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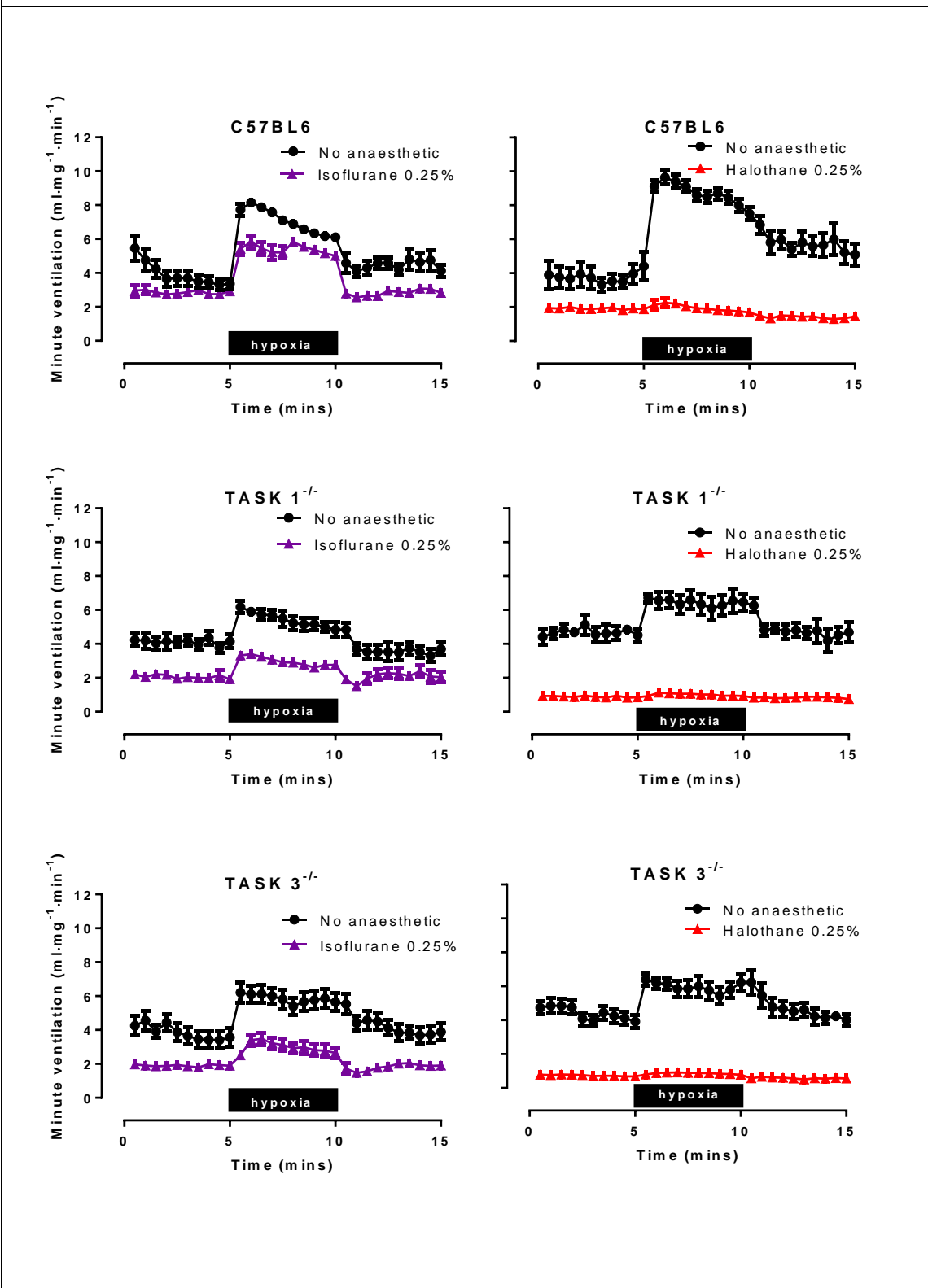
In the wildtype, anaesthetic agent had a significant influence on the depression of the AHVR (Figure 3.9; ANOVA,  $p=0.04$ ). The order of potency of the depression of the AHVR was established as halothane>isoflurane (post-hoc  $p=0.04$ ) in the wildtype.

In all genotypes, the highest doses of halothane (0.2-0.4 MAC) virtually abolished AHVR. However, at these similar doses, isoflurane only depressed AHVR by ~60%. Isoflurane appears the least depressive in the TASK 3<sup>-/-</sup> mice (Figure 3.10).

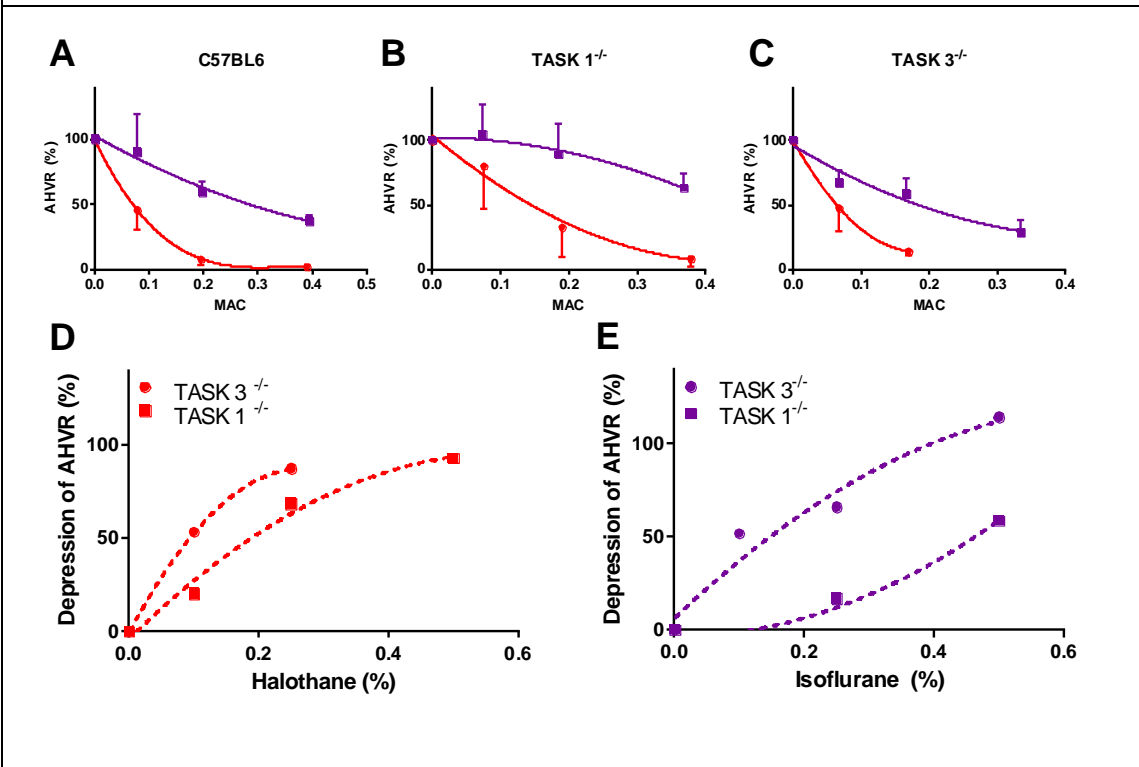
#### **Influence of anaesthetics on responses to CO<sub>2</sub>**

Concentration of anaesthetic had a significant influence on ventilatory response to CO<sub>2</sub> ( $p=0.007$ ), and halothane was again more depressive of this response than isoflurane ( $p=0.039$ ). There was a genotype influence on the magnitude of this response ( $p<0.001$ ) with the C57BL6 response in anaesthetic greater than the TASK 1<sup>-/-</sup> response which in turn had a greater magnitude than the TASK 3<sup>-/-</sup> response (Figure 3.11).

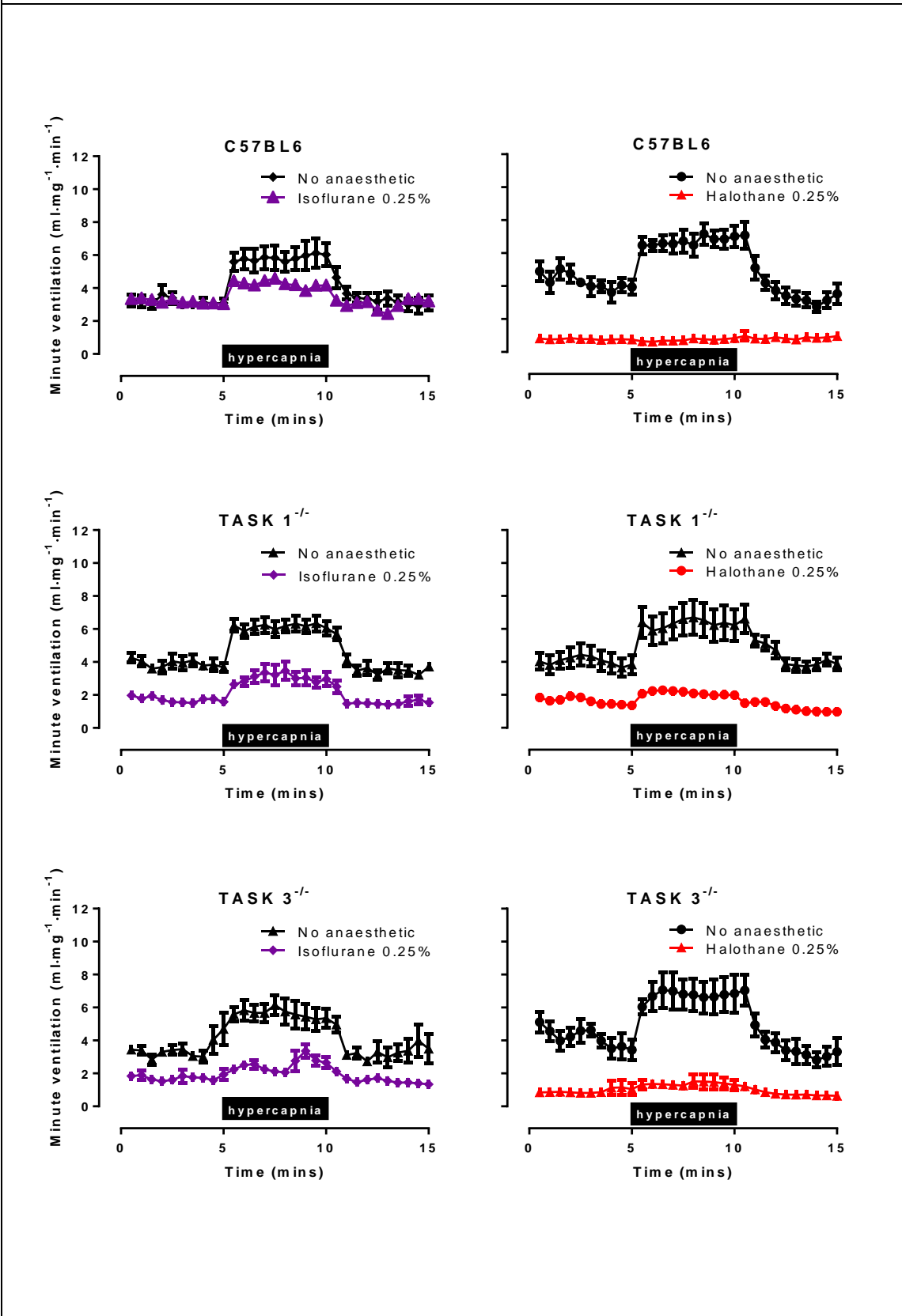
**Figure 3.9** Influence of volatile anaesthetics (halothane red and isoflurane purple) on the ventilatory response to isocapnic hypoxia (10% O<sub>2</sub> with 3% CO<sub>2</sub>).



**Figure 3.10** Panels **A-C** show how the agents influence AHVR across three genotypes. In all species, halothane is more depressive than isoflurane (ANOVA,  $p < 0.001$ ). Panels **D** and **E** are the same data expressed as % maximum depression by agent in the wildtype. This shows how a single agent differentially or does not influence AHVR by genotype. Thus, halothane equally and profoundly influences AHVR in both genotypes (panel **D**). However, isoflurane depresses AHVR in the TASK 3<sup>-/-</sup> genotype much more than the TASK 1<sup>-/-</sup> genotype ( $p = 0.006$ ). Concentrations of anaesthetics (halothane red and isoflurane purple) are plotted as % volume and minimum alveolar concentration, adjusted for the appropriate genotype (Sonner et al., 2000; Linden et al., 2006; Linden et al., 2007; Sonner et al., 2007).



**Figure 3.11** Influence of volatile anaesthetics (halothane red and isoflurane purple) on the ventilatory response to hypercapnia (4% CO<sub>2</sub> with euoxia).



## **Discussion**

The main findings of this chapter are: (1) ventilatory responses to hypoxia are only evident in background isocapnia, and not poikilocapnia; (2) TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup>, TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> and TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> mice have diminished acute ventilatory responses to hypoxia and hypercapnia as compared with wildtypes; (3) TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> mice have altered baseline ventilatory patterns in euoxia, exhibiting more tachypnoea than wildtypes; (4) the agents halothane and isoflurane depress the AHVR in wildtypes with an order of potency that matches previous reports in humans and rats (i.e., halothane > isoflurane); (5) halothane and isoflurane depress AHVR in both TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup>, but whereas these genotypes are equally sensitive to halothane, the former are more sensitive to isoflurane.

## **Ventilatory responses of wildtype and knockout mice to hypoxia**

I have demonstrated that an ablation of TASK 1, TASK 3 or both causes mice to have diminished ventilatory responses to hypoxia and hypercapnia as compared with the wildtype. This is contrary to the findings of Trapp et al. (2008) who found reduced responses in only TASK 1<sup>-/-</sup> mice (29% increase from baseline vs 64% in wildtypes). The difference in techniques is that here isocapnic hypoxia was employed. Even in the poikilocapnic protocol it a near-absence of AHVR was found in both knockouts (Figure 3.6) whereas Trapp et al. (2008) reported a 29% and ~66% increase from baseline ventilation with hypoxia using poikilocapnia in TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> respectively. In my hands, this near-absence of ventilatory responses meant poikilocapnic hypoxia was not amenable for meaningful further study of hypoxic responses. The

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reasons for these differences of magnitude of responses between Trapp's study and this in poikilocapnia are unclear.

Studies on isolated glomus cells of these knockout strains do not shed light on these differences. Turner and Buckler (2013) note that the increase in glomus cell  $[Ca^{2+}]_i$  evoked by hypoxia was robust across genotypes. However, in their single channel electrophysiology experiments, cells expressing TASK 1 only (i.e., TASK 3<sup>-/-</sup>) respond more robustly than those that express TASK 3 only (TASK 1<sup>-/-</sup>). The protocol used in vitro by (Turner and Buckler, 2013) rigorously controlled for CO<sub>2</sub>, using a bicarbonate buffered superfusate bubbled with 5% CO<sub>2</sub>. Analogously in my study, the isocapnic AHVR was more robust in animals expressing only TASK 1 although these differences did not reach statistical significance (AHVR of 65% in TASK 3<sup>-/-</sup> vs TASK 1<sup>-/-</sup> 41%,. p = 0.83). The relative hypoxia responses across these studies is presented in Table 3.9.

There are several reasons why single cell work may not reflect whole-animal responses. The hypoxic stimulus at single cell level is super-maximal, and a very strong stimulus could overcome and obliterate any genotype differences. Cell-cell interactions are lost in the isolated cell preparation, but contribute to the whole organ response (Nurse, 2014). Finally - and perhaps most influentially - a central nervous system role for TASK channels in general in the control of respiration independent of chemosensitivity may be important (Gestreau et al., 2010). Thus in the knockout mice, central control of respiration is affected which in turn might influence hypoxic responses. Some evidence for this is the changed baseline ventilatory pattern in the knockout (See Appendix A).

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**Table 3.9** Summary of relative responsiveness to hypoxia across levels of organisation (+ least, ++ intermediate, +++ most responsive). For Turner and Buckler (2013)  $[Ca^{2+}]_i$  response to hypoxia and single channel electrophysiology, for Trapp et al. (2008) and the present study whole animal plethysmography. This shows that the studies broadly agree, with the exception of at the level of calcium recordings: this is discussed by Turner and Buckler (2013).

	Wildtype	TASK 1 <sup>-/-</sup> animals/cells (TASK 3 channels present only)	TASK 3 <sup>-/-</sup> animals/cells or (TASK 1 channels present only)
This study	+++	+	++
Trapp et al. (2008)	+++	+	+++
Turner and Buckler (2013)(channel)	+++	+	+++
Turner and Buckler (2013)(calcium)	+++	++	+

These results are consistent with the conclusion of Kim et al. (2009) that the predominant oxygen sensitive conductance in rat and mouse glomus cells at the highly reduced single channel level is the TASK 1/3 heteromultimer (Kim et al., 2009; Turner and Buckler, 2013). Therefore it is rational that in the whole animal, loss of either functional allele would diminish oxygen sensitivity. This was the observation here. By studying the TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup>, I sought to explore whether this heterozygote mutant animal would have an intermediate, or even normal ventilatory phenotype. With a single normal allele of TASK 1 and TASK 3, and thus genetically possessing the machinery to produce the full array of normal TASK isoforms, it might be reasonably expected that TASK 1/3 heteromultimers would form. The finding that the double heterozygote has a diminished AHVR similar to all the other mutant allele combinations (Figure 3.6)

rather than an intermediate phenotype is of interest. The disruption of a wildtype gene by a functional mutant gene has long been recognised in the field of transgenics (Herskowitz, 1987). It has since been described in the  $K_{2P}$  channel family both in vivo (Bando et al., 2014) and in vitro (Berg et al., 2004; Veale et al., 2014) and raises the possibility that here, if the mutant sequences are transcribed a dominant negative protein could form. The presence of either aberrant TASK 1 or TASK 3 protein would thus give a false impression of the true function of each normally formed isoform. For example, if mutant TASK 1 ( $Kcnk3^{Tm1Sgb}$ ) were to exhibit the same dominant negative effect on TASK 3 as  $Kcnk3^{Y191F}$  (a mutation of TASK 1) has (Berg et al., 2004), normal TASK 3 function could be masked in TASK 1 knockout mice. Similarly,  $Kcnk9^{G95E}$  (Brenner and O'Shaughnessy, 2008), has been used as a way to knock down normal TASK 3 function, a similar effect of  $Kcnk9^{Wwis1}$  studied here could result in the an abnormal phenotype, even in heterozygote animals.

### **Ventilatory responses of wildtype and knockout mice to CO<sub>2</sub>**

I report here a depressed magnitude of response to hypercapnia and reduced sensitivity to CO<sub>2</sub> again across all TASK mutant genotypes (TASK 1<sup>-/-</sup>, TASK 3<sup>-/-</sup>, TASK 1<sup>+/-</sup>/TASK 3<sup>+/-</sup> and TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup>) as compared with wildtype (Figure 3.7, Figure 3.8 and Table 3.8). This in contrast to some previous studies that suggest normal ventilatory responses to CO<sub>2</sub> in knockouts (Mulkey et al., 2007; Trapp et al., 2008). The methodological difference that might explain these results is that in those other studies CO<sub>2</sub> was introduced in a graded “ramp” type protocol more likely to assess the central “slow” response to hypercapnia (Pedersen et al., 1999). It has long been established that the temporal

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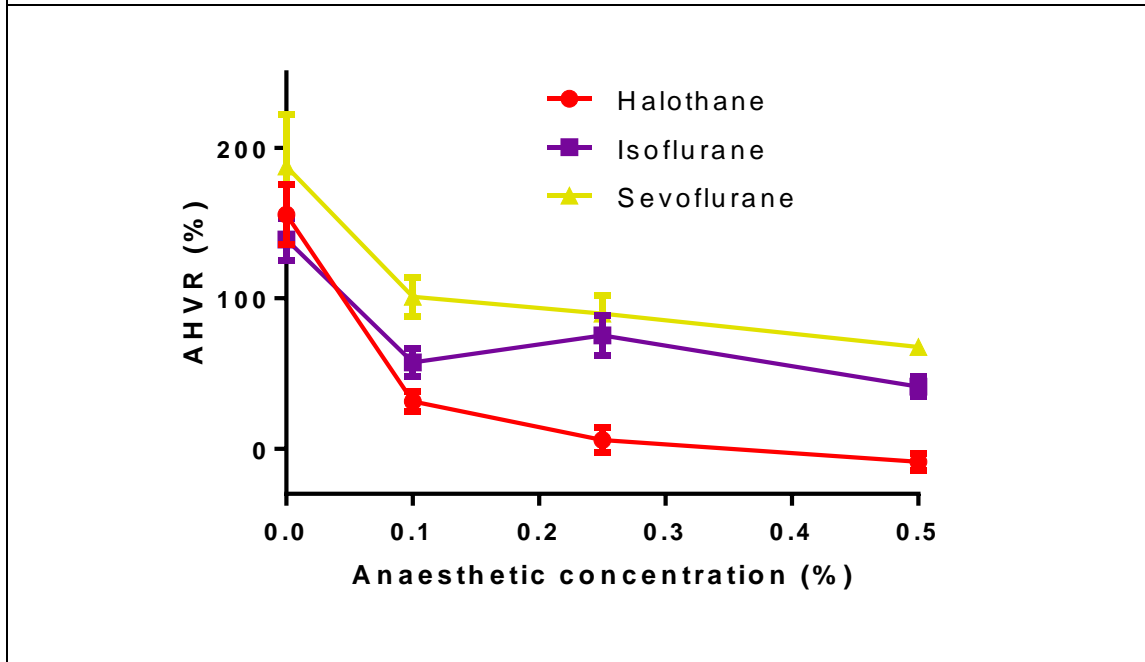
characteristics of how a hypercapnic response is presented will influence the magnitude of response (Dahan et al., 1990; Gozal et al., 1994). A gradual increase in CO<sub>2</sub>, akin to Read's rebreathing method, may overestimate CO<sub>2</sub> sensitivity; the mechanism underlying this response may in turn be related to how the brain vasculature responds to CO<sub>2</sub>, and hence determines brain tissue pCO<sub>2</sub> (Pandit et al., 2008). The square wave "step" protocol is therefore unique in the assessment of CO<sub>2</sub> response in TASK channel knockout animals. The finding of both a depressed magnitude of response to hypercapnia, and reduced sensitivity to CO<sub>2</sub> in both TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> strains further emphasise the importance of this multimodal TASK 1/3 chemosensor at the level of the carotid body. This result is as I expected, as TASK is well established as being sensitive to CO<sub>2</sub> (Trapp et al., 2008; Ortega-Saenz et al., 2010).

#### **Anaesthetic effects on AHVR of wildtype mice**

As anticipated, halothane and isoflurane depressed the AHVR (Figure 3.9) in the wildtype mouse. This follows the characteristic order of potency halothane > isoflurane which has been described for K<sub>B</sub> (native TASK) channels (Pandit et al., 2010b), isolated glomus cells (Pandit and Buckler, 2009; Pandit et al., 2010b), intact rats (Karanovic et al., 2010) and humans (Pandit, 2002). Additionally in wild type mice (but not KOs) I examined the effects of sevoflurane, an agent that in the Pandit (2005) analysis and the Karanovic et al. (2010) experiment (for rats) is probably the least depressive. Figure 3.12 shows that this agent is indeed less depressive of AHVR in wildtypes than the other agents. This homology of behaviour between humans, rats and now mice

provides further validation for the C57BL6 mouse as a model for the anaesthetic depression of AHVR, and creates the possibility of interrogating these mechanisms using knockouts of other implicated candidate genes (e.g. TREK-1 or BKCa).

**Figure 3.12** AHVR (%  $\pm$  SEM) over a range of anaesthetic concentrations in C57BL6 mice (n=8 for each agent)



### Anaesthetic effects on AHVR in knockout mice

It is presented here that TASK channels play a role in the anaesthetic modulation of the hypoxic ventilatory response. To my knowledge, this is the first demonstration of these effects in TASK-knockout mice. The key finding is that TASK channels appear central to the differential effects of the volatile anaesthetic agents halothane and isoflurane. However, detailed consideration is needed of the effects I report.

Previous work suggests that the TASK 1 subunit is insensitive to isoflurane (Putzke et al., 2007). I had therefore expected mice lacking TASK 1 (TASK 3<sup>-/-</sup>)

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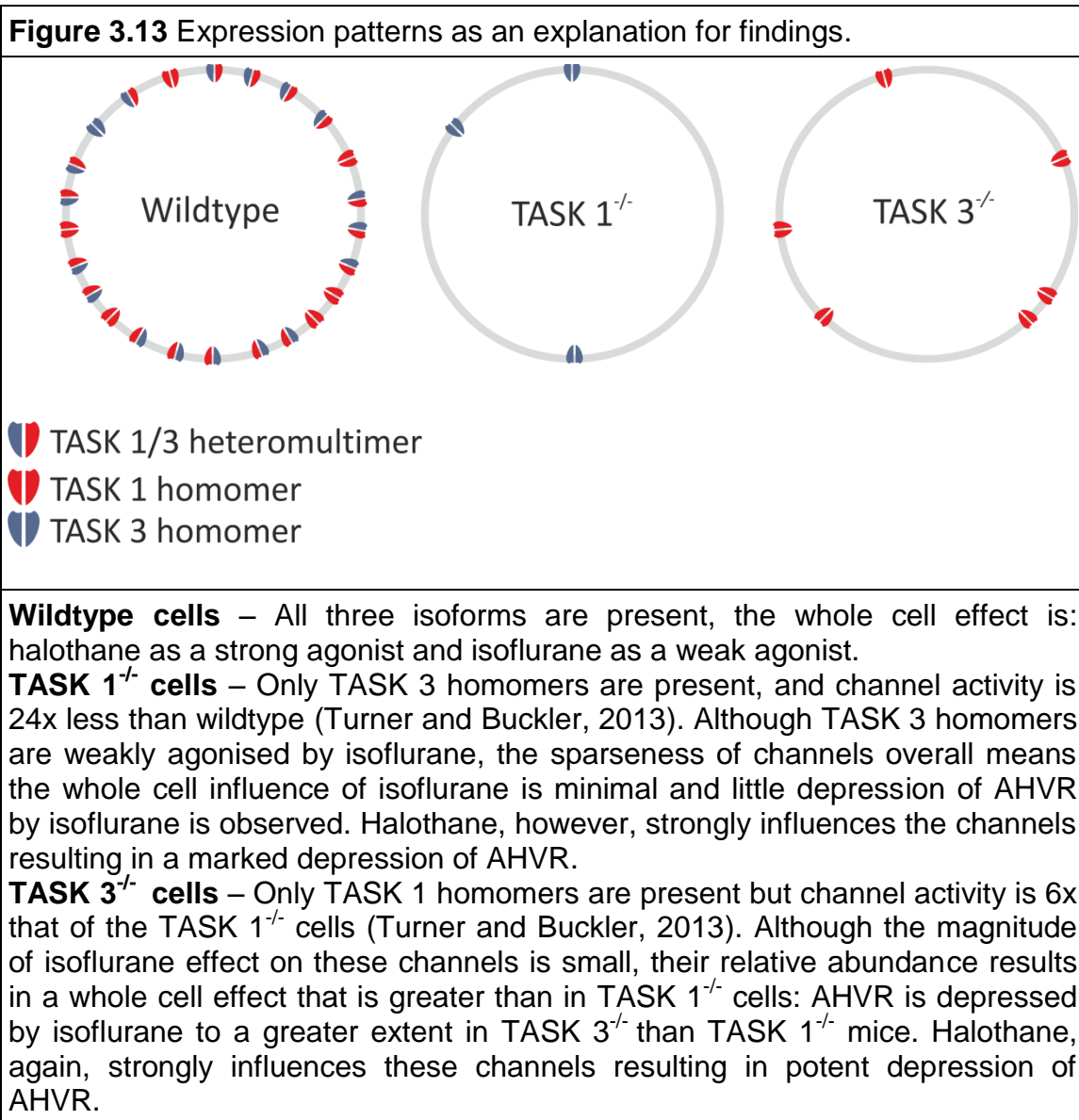
to show little or no difference vs wild types in their response to isoflurane. However, it was found that TASK 3<sup>-/-</sup> mice were less sensitive to AHVR depression by isoflurane than wildtype or TASK 1<sup>-/-</sup>.

This counterintuitive result may be explained by the complex expression of K<sub>B</sub> channels (Kang et al., 2004), emerging evidence that this family of proteins are regulated by SUMOylation (Plant et al., 2012), and the fact that these are genomic knockouts and therefore may have adapted during growth. Total K<sub>B</sub> channel expression appears diminished in both knockout mouse strains in single channel recordings of isolated glomus cells (Turner and Buckler, 2013). Total K<sub>B</sub> channel activity in TASK 3<sup>-/-</sup> glomus cells is much greater (sixfold) than that of TASK 1<sup>-/-</sup> cells (Turner and Buckler, 2013). Since therefore, in TASK 1<sup>-/-</sup> glomus cells, there are fewer functional channels, their influence on membrane potential is limited. In turn this also explains the small AHVR in TASK 1<sup>-/-</sup> animals. (Figure 3.2).

Furthermore, isoflurane (which has an intrinsically weaker action than halothane in opening the channels (Pandit et al., 2010b)) results in a relatively feeble depression of AHVR. In contrast, halothane (which has a strong effect on channel opening) results in a greater depression of AHVR (Figure 3.10)

In TASK 3<sup>-/-</sup> cells there are overall more functional K<sub>B</sub> channels than in TASK 1<sup>-/-</sup> (Turner and Buckler, 2013), but fewer overall than the wild type. So there is a smaller AHVR than in wildtypes, but a larger AHVR than in the TASK 1<sup>-/-</sup>. (consistent with my findings – Figure 3.6) Because this greater number of channels makes a greater contribution to membrane potential, there results a greater depression of AHVR by isoflurane than occurs in TASK 1<sup>-/-</sup> animals,

even though in isolation the remaining TASK 1 channels are somewhat insensitive to isoflurane (Berg et al., 2004). A model outlining this explains most, if not all of these observations (Figure 3.13).



## **Limitations**

It is usual in human studies to employ dynamic end-tidal forcing and a multifrequency binary sequence of gas switching to dissect the peripheral (fast) component of the response to CO<sub>2</sub> from the central (slow) response (Pedersen et al., 1999; Teppema and Dahan, 2010). This is not feasible in unrestrained mouse plethysmography, so any influence of anaesthetic on the hypercapnic response would be a composite of peripheral and central responses, and is therefore very difficult to interpret in the context of interest. I have used the technique of deriving peripheral contribution to CO<sub>2</sub> response as a subtraction of the response in hyperoxia from that in hypoxia (Figure 2.16).

The common feature of knockout strains studied here is the lack of the TASK 1/3 heteromultimer, with the exception of the TASK 1<sup>+/-</sup>/3<sup>+/-</sup>, which may be able to form these channels. A material difference between the TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> has been demonstrated with respect to anaesthetic depression on AHVR which can be inferred to be due to the differential expression of homomeric channels in the two strains. However, it does not exclude compensatory effects in the expression of the remaining TASK channel in each knockout. It would be highly desirable to repeat the methodology in double knockouts, but I was limited by animal welfare concerns. At times, the TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> appeared to be aesthenic in hypoxia (consistent with their marked depression of ventilation in poikilocapnia). It was felt by me and the Named Animal Care and Welfare Officer that the addition of an anaesthetic would exceed the severity threshold that was set prior to experimentation, the likely result being animal death. This genotype has hitherto been rarely studied, and not at all with

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respect to anaesthetic effects. Mulkey et al. (2007) found genomic double knockout mice to have an “unremarkable respiratory and neurological phenotype”, but presented no results. I would have to disagree with that overview.

There is a possibility that a distinct (i.e., non-TASK) hypoxia sensitive conductance may be expressed in the absence of TASK channels and the methodology here could not exclude this possibility. This is supported by recently published work from this laboratory which observed a non-TASK conductance, possibly TREK or TRAAK, in patch clamp experiments on isolated carotid body type-1 cells from knockout mice (Turner and Buckler, 2013), which is not normally observed in wildtype cells. Since hypoxia-stimulated calcium influx was the same in knockout glomus cells as in wildtype cells (Turner and Buckler, 2013), this implies that germline TASK 1/3 deletion must have been compensated for at the cellular level. The effect of volatile anaesthetics on AHVR in double knockouts in vivo would therefore be complex to interpret. If they had exhibited a normal hypoxic response (as suggested by Mulkey et al. (2007)), it could equally be expected that they were resistant to volatile depression of AHVR (given their lack of TASK 1 and TASK 3), or susceptible due to an upregulated non-TASK (yet anaesthetic sensitive) channel such as TREK. Given the lack of a significant AHVR in the TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> animals, this was not explored.

Exposure to anaesthetic agents undoubtedly influences the central respiratory control centres (Stuth et al., 2012); depressed conscious level of the animal is inevitable when exposed to anaesthesia and this in turn will further inhibit

ventilatory responses. In other words, central effects of anaesthesia limits the concentrations that can be used that avoid a central depressive action. It is known that hypoxic ventilatory responses are effected by level of arousal, and this is also agent-dependent (Sarton et al., 1996; Pandit et al., 2004). I did not specifically control for conscious level (e.g. with concomitant assessment of MAC by loss of righting reflex) as this would have interfered with the measurement of AHVR. It is therefore not possible from this study to dissect the central from peripheral effect of the volatile agents, and TASK channels are known to be expressed widely throughout the nervous system (Medhurst et al., 2001). Some groups have taken the approach of examining nerve recordings to better locate the anatomical site of anaesthetic depression of AHVR (Karanovic et al., 2010), however these studies require surgical doses of anaesthesia, or a protocol that decorticates the model species, both confounders in their own right.

### **Implications**

There are a number of broader conclusions of this work:

1. All species of TASK channel assembly appear important in the generation of a normal AHVR and CO<sub>2</sub> response. Loss of either TASK 1 or TASK 3 (and thus thereby the heteromultimer) has a significant functional consequence for hypoxic and hypercapnic responses.
2. The volatile agents isoflurane and halothane have discrete and deleterious effects on chemosensation regardless of which TASK channels are expressed. As such, to prevent the adverse ventilatory effects of anaesthetics, it would desirable to develop antagonists for

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the action of these agents at all of the channel subtypes. Current pharmaceutical agents that stimulate breathing (e.g. doxapram) have significant off-target side effects due to their lack of channel specificity.

3. In the development of novel hypnotic anaesthetic agents, it would be desirable for them to act independently of TASK channels, in order better to preserve the hypoxic/hypercapnic chemoreflexes. This could be part of a high throughput electrophysiological screening process of candidate compounds, or perhaps using the methodologies described here.

**Chapter 4:**

**DEPRESSIVE ACTIONS OF PROPOFOL ON CAROTID**

**BODY GLOMUS CELL CHEMOSENSATION**

## **Overview**

Propofol is a commonly used general anaesthetic drug, employed also for sedation in critical care (Jacobi et al., 2002; Barr et al., 2013) and procedural settings (Riphaus et al., 2013). The recent 5<sup>th</sup> National Audit Project (NAP5) in the United Kingdom reported that it was used for 86% of all anaesthetic inductions and in 8% of cases as total intravenous anaesthesia for maintenance (Pandit and Cook, 2014; Sury et al., 2014). Considerable evidence suggests that propofol exerts its hypnotic effect primarily through modulation of GABA-A channels (Krasowski et al., 1998; Jurd et al., 2003; Franks, 2008), and novel binding sites and modelling of this receptor re-inforce this suggestion (Ruesch et al., 2012; Franks, 2015).

Quite separate from their hypnotic effects, it is well established that volatile anaesthetic agents, even in low dose and as a side-effect, considerably blunt ventilatory response to both hypoxia and hypercapnia (Knill and Gelb, 1978; Dahan et al., 1994; Sollevi and Lindahl, 1995; van den Elsen et al., 1995; Pandit et al., 1999b; Pandit et al., 1999a; Pandit, 2002, 2005). Propofol has been shown to have a similar effect, (Blouin et al., 1993; Nagyova et al., 1995; Akada et al., 2008). With an expanding number of procedures carried out under propofol sedation understanding the mechanism by which this occurs is important.

Although the anatomic and molecular sites of action for propofol's effect within the respiratory system are not known, the carotid bodies are prime focus of interest. The type I (glomus) cells of these organs respond by an increase in intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) to hypoxia and hypercapnia (Buckler and Vaughan-

#### *Chapter 4 - Depressive actions of propofol on carotid body glomus cell chemosensation*

Jones, 1994a; Buckler and Vaughan-Jones, 1994b; Dasso et al., 2000). The  $\text{Ca}^{2+}$  influx causes vesicles containing neurotransmitter to fuse with the type I cell membrane (Kim and Kang, 2014). There are several candidate neurotransmitters that activate the postsynaptic (afferent nerve terminal) membrane, amongst which are: ATP (Fearon et al., 2003a; Zhang et al., 2012) and acetylcholine (Fitzgerald et al., 1999; Shirahata et al., 2007). GABA is thought to provide presynaptic autoregulatory feedback during hypoxia (Fearon et al., 2003b) and adenosine presynaptic autostimulation (Vandier et al., 1999). Neurotransmitter binding leads to synaptic stimulation of the terminals of the carotid sinus nerve (Kåhlin et al., 2014). This process is highly regulated by neurocrine, autocrine and paracrine mechanisms (Nurse, 2014). Excitation of the afferent terminals of the glossopharyngeal nerves sends signals to the breathing centres of the brainstem, in turn to trigger reflex hyperventilation (Weir et al., 2005). The detailed mechanisms of oxygen ( $\text{O}_2$ ) and carbon dioxide ( $\text{CO}_2$ ) sensing are complex and contentious, though there is consensus that the background potassium ( $\text{K}^+$ ) conductance mediated primarily by TASK (TWIK-related acid sensing potassium) channels, is modulated by these stimuli such that membrane depolarisation occurs and causes voltage-gated calcium influx into glomus cells (Buckler, 2015).

This chemoreflex pathway therefore could theoretically be inhibited by anaesthetics acting at the carotid body itself (e.g., via interference of any of the mechanisms above) or along the reflex arc more centrally within the brain (e.g., through the general depressant action of anaesthetics on brain function. The brain itself, however, is not directly sensitive to hypoxia in an adaptive way). At

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the carotid body, there are broadly two potential sites at which excitation by hypoxia or CO<sub>2</sub> could be inhibited: either by an action on one or more of the cellular mechanisms detecting/transducing these stimuli, or by an action at the nerve terminal (e.g., preventing neurotransmitter from activating the neural afferents). For volatile anaesthetics, a leading candidate mediator of depressive effects on hypoxia/CO<sub>2</sub> response is the background potassium/TASK channels, which (possibly uniquely) appears to be sensitive to all of: hypoxia, CO<sub>2</sub> and halothane (Buckler et al., 2000), and which subsequently has been also found to be influenced by other volatile agents such as isoflurane and sevoflurane (Pandit et al., 2010b). However, the TASK channel appears to be insensitive to propofol (Putzke et al., 2007).

The possibility that propofol inhibits the hypoxic chemoreflex at the nerve terminal was explored by Jonsson et al. (2005) who used an isolated perfused rabbit carotid body preparation, recording from the afferent nerve. Propofol reduced both hypoxia- and nicotine-induced nerve activation. They propose propofol was acting via nicotinic postsynaptic receptors. Notably, bicuculline (a GABA receptor antagonist) did not have any additional effect in combination with propofol, suggesting it was unlikely that GABA was involved at the postsynaptic terminal. These results are consistent with observations that the carotid body secretes acetylcholine that acts on postsynaptic nicotinic receptors, and that curare-like neuromuscular blocking drugs also inhibit the hypoxic response, acting synergistically with anaesthetic agents (Eriksson, 1999; Jonsson et al., 2004).

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One limitation of the Jonsson et al. (2005) study was that, being a preparation of carotid body and nerve fibre, a possible effect of propofol (and of the putative antagonists to its effect) directly on the type I cells could not be excluded. Although propofol may not act on TASK channels (Putzke et al., 2007), there are other parts of the O<sub>2</sub>- and CO<sub>2</sub>-sensing apparatus on which propofol might theoretically act, as yet unexplored. It appears that historically, the effects of propofol on chemoreflex drive have been studied only in humans (Nagyova et al., 1995), isolated perfused carotid body preparations (Jonsson et al., 2004; Jonsson et al., 2005) or intact, anaesthetised animals (with recording from phrenic nerve (Ponte and Sadler, 1989a)). A possible direct action on glomus cells appears uninvestigated. Yet, nicotinic receptors are expressed on glomus cell membranes, and their activation by nicotine or acetylcholine induces a rise in [Ca<sup>2+</sup>]<sub>i</sub> (Dasso et al., 1997), probably by causing depolarisation that opens voltage-gated Ca<sup>2+</sup> channels. Furthermore, nicotinic receptors are suggested (in addition to GABA receptors) as a putative molecular target for the hypnotic effects of propofol (Flood et al., 1997; Violet et al., 1997; Tassonyi et al., 2002).

I therefore wished to investigate the possibility that propofol exerts an inhibitory effect directly on the type I O<sub>2</sub>- and CO<sub>2</sub>-sensing cells of the carotid body, in a preparation devoid of connecting neural tissue. A direct effect of propofol on the glomus cell would add to its range of possible mechanisms of action. Or, being the site most 'proximal' in the chemoreflex pathway it might make putative actions at other distal sites somewhat redundant as explanations for the drug's depressive effect on chemosensing.

## *Chapter 4 - Depressive actions of propofol on carotid body glomus cell chemosensation*

From the results of these earlier studies, I hypothesise that:

- (1) Propofol will depress the polymodal chemoexcitation of the carotid body glomus cells to hypoxia, CO<sub>2</sub> and potassium.
- (2) Propofol will depress the nicotinic stimulation of glomus cells, as will vecuronium.
- (3) GABA will similarly depress the glomus cell response to hypoxia.
- (4) Antagonising GABA-A and/or GABA-A will conversely augment the chemosensory response to hypoxia.
- (5) TASK channels in glomus cells will not be effected by propofol.

### **Methods**

All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

### **Cell isolation**

Carotid bifurcations were dissected in situ from neonatal Sprague-Dawley rats (P11-14) under terminal isoflurane anaesthesia and placed in ice-cold saline. Animals were supplied by Harlan (Blackthorn, Oxfordshire, UK). Microdissection of the carotid body was carried out ex vivo, and after enzymatic treatment the tissue was triturated to isolate individual cells in suspension as previously described (Buckler and Vaughan-Jones, 1993; Buckler et al., 2000). Primary cell cultures were plated onto coverslips and incubated for two hours prior to loading with indo-1, AM dye for 60 minutes. Primary cell cultures were pooled and also prepared for use in unrelated experiments concurrently.

### **Calcium imaging**

Glomus cells were excited at a wavelength of 340 nm by filtered light from an arc lamp. In epifluorescence mode, emission light was split and measured at dual emission peaks of 405 nm and 495 nm by two trialkali photomultiplier tubes (Thorn EMI). Intracellular calcium concentration ( $[Ca^{2+}]_i$ ) was estimated using the calibrated ratio of the fluorescence intensities as described in the General Methods chapter.

### **Hypoxia experiments**

Each cell was sequentially superfused with 37°C Tyrode's solution (in mM: 117 NaCl, 2.5 CaCl<sub>2</sub>, 4.5 KCl, 1 MgCl<sub>2</sub>, 23 NaHCO<sub>3</sub>, 11 glucose) equilibrated with 5% CO<sub>2</sub> in balanced air (euoxia) and 5% CO<sub>2</sub> in balanced N<sub>2</sub> (severe hypoxia) or 5% CO<sub>2</sub>, 1% O<sub>2</sub> in balanced N<sub>2</sub> (mild hypoxia). Severe hypoxia (0% O<sub>2</sub>) was measured using a fluorescence quenching optode, at < 3 mmHg O<sub>2</sub> in the perfusion chamber. This was designed to elicit robust activation of cells and probably mimick tissue conditions during whole body exposures to end-tidal hypoxia of ~50 mmHg (Acker et al., 1971). In contrast, mild hypoxia was measured at ~15 mmHg O<sub>2</sub>, and was used in the case where it was expected that an augmentation of cellular response to hypoxia may occur (e.g. with GABA-B antagonism; see below and Fearon et al. (2003b)).

### **Hypercapnia experiments**

Cells were sequentially superfused with euoxia as previously described, designed to mimic typical arterial CO<sub>2</sub> tensions in the physiological range. Hypercapnic exposure consisted of a rapid switch to warmed Tyrode's solution equilibrated with 20% CO<sub>2</sub>, 80% air, with sodium chloride substituted with sodium bicarbonate and buffered to pH 7.4 at 37°C (isohydric hypercapnia).

### **High $[K^+]_o$ experiments**

Each cell was challenged with the standard hypoxic stimulus as a positive control for identifying functional glomus cells, prior to superfusion with a Tyrode's solution modified to contain 30 mM KCl (Table 4.1). This condition chemically depolarises the membrane, initiating voltage gated calcium entry. At this level of  $[K^+]_o$ , the Nernst equation predicts the membrane to depolarize to approximately -41 mV (i.e. the calculated equilibrium potential for  $K^+$ , assuming that  $[K^+]_i$  is 140 mM). Under these depolarized conditions, activation of voltage-gated  $Ca^{2+}$  channels causes an increase in  $[Ca^{2+}]_i$  in the glomus cell (Overholt and Prabhakar, 1997).

### **Electrophysiology experiments**

Cell-attached patch clamp electrophysiology was performed using an Axopatch 200B and borosilicate glass capillaries coated with Sylgard (Dow Corning, Senefte, Belgium) fire-polished immediately before use. Cell-attached filling solution contained 140 mM KCl, 1 mM  $MgCl_2$ , 1 mM EGTA, 10 mM HEPES, 10 mM tetraethylammonium and 5 mM 4-aminopyridine at pH 7.4 at 37°C. Recordings were conducted in modified Tyrode's solution (in mM: 100 KCl, 21.5 NaCl, 23  $NaHCO_3$ , 11 glucose), to fully depolarise and therefore stabilise the cell membrane. The membrane patch was held at a pipette potential of +80 mV. Membrane current was filtered at 2 kHz and digitized at 20 kHz prior to data acquisition with Spike2 software. The main conductance state for each recording was defined using an all points histogram, and the threshold for opening set at 50% of this value. Multiple openings were defined as current of 150%, 250%, 350%, etc., of the main conductance state, as multiple channels

were often present in a patch, single-channel activity was quantified using  $NP_{open}$  as described in Chapter 2 – General Methods (Figure 2.9).

**Table 4.1** Modified Tyrode's solution for exciting glomus cells with potassium 30 mM.

Ingredient	Final concentration
KCl	30 mM
NaCl	91.5 mM
NaHCO <sub>3</sub>	23 mM
CaCl <sub>2</sub>	2.5 mM
MgCl <sub>2</sub>	1 mM
Glucose	11 mM

### Drugs

All drugs were appropriately reconstituted daily prior to dissolution in Tyrode's solution. Propofol 1% (Fresenius, Runcorn, Cheshire, UK) was used in concentrations of 10-500  $\mu$ M, and the propofol vehicle Intralipid 10% (Fresenius) was used as a negative control. GABA (Sigma-Aldrich, Gillingham, Dorset, UK) was applied in concentrations of 5  $\mu$ M as a synaptic concentration to restore tonic GABA activity or 1 mM to evoke maximal GABA activity. Muscimol 50  $\mu$ M (Sigma-Aldrich) was used to selectively activate GABA-A, which was in turn antagonised to exclude tonic activity by bicuculline 100  $\mu$ M. Baclofen 50  $\mu$ M (Abcam, Cambridge, Cambridgeshire, UK) was used to activate and 5-aminovalleric acid 100  $\mu$ M (5-AVA, Sigma-Aldrich) to inhibit GABA-B in isolation. Nicotine 300  $\mu$ M (Sigma-Aldrich) was used as a selective nAChR agonist, vecuronium 10  $\mu$ M (Abcam) as a competitive, non-selective antagonist

of nAChR, while methyllycaconitine 50  $\mu$ M (MLA, Abcam) was used as a selective nAChR antagonist to ensure blockade of all (including  $\alpha$ 7 homomeric) nAChRs where no effect of vecuronium was observed.

## **Results**

### **Effect of propofol on glomus cell response to hypoxia**

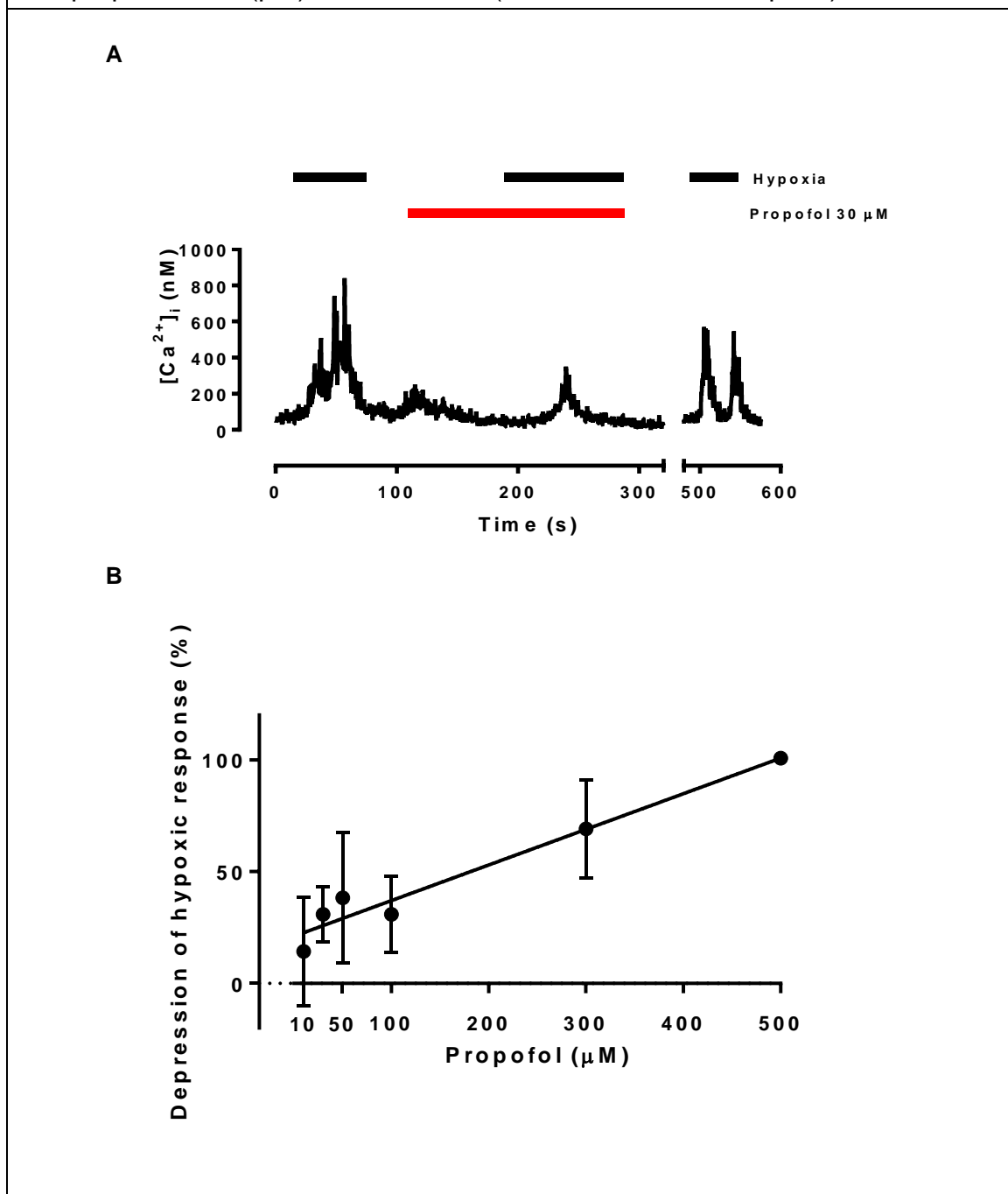
Propofol had a statistically significant depressive influence on euoxic  $[Ca^{2+}]_i$  from  $98 \pm 9$  nM (n= 42), to  $66 \pm 5$  nM (n=42),  $p < 0.001$ . At a dose of 30  $\mu$ M (corresponding to a clinical concentration of  $\sim 5$   $\mu$ g/ml) propofol reduced the hypoxia-induced rise in  $[Ca^{2+}]_i$  response from  $437 \pm 133$  nM to  $255 \pm 153$  nM (n=9;  $p = 0.015$ , Figure 4.1A). More strikingly, propofol caused a significant dose-dependent depression of the magnitude of hypoxia-induced rise in  $[Ca^{2+}]_i$  (Figure 4.1B;  $p = 0.043$  for dose, ANOVA).

In control experiments, Intralipid 10% into which propofol is suspended in its clinical presentation, was used but had no effect on hypoxia induced rise in  $[Ca^{2+}]_i$  ( $183 \pm 48$  vs  $159 \pm 63$  nM; n=11,  $p = 0.469$ ).

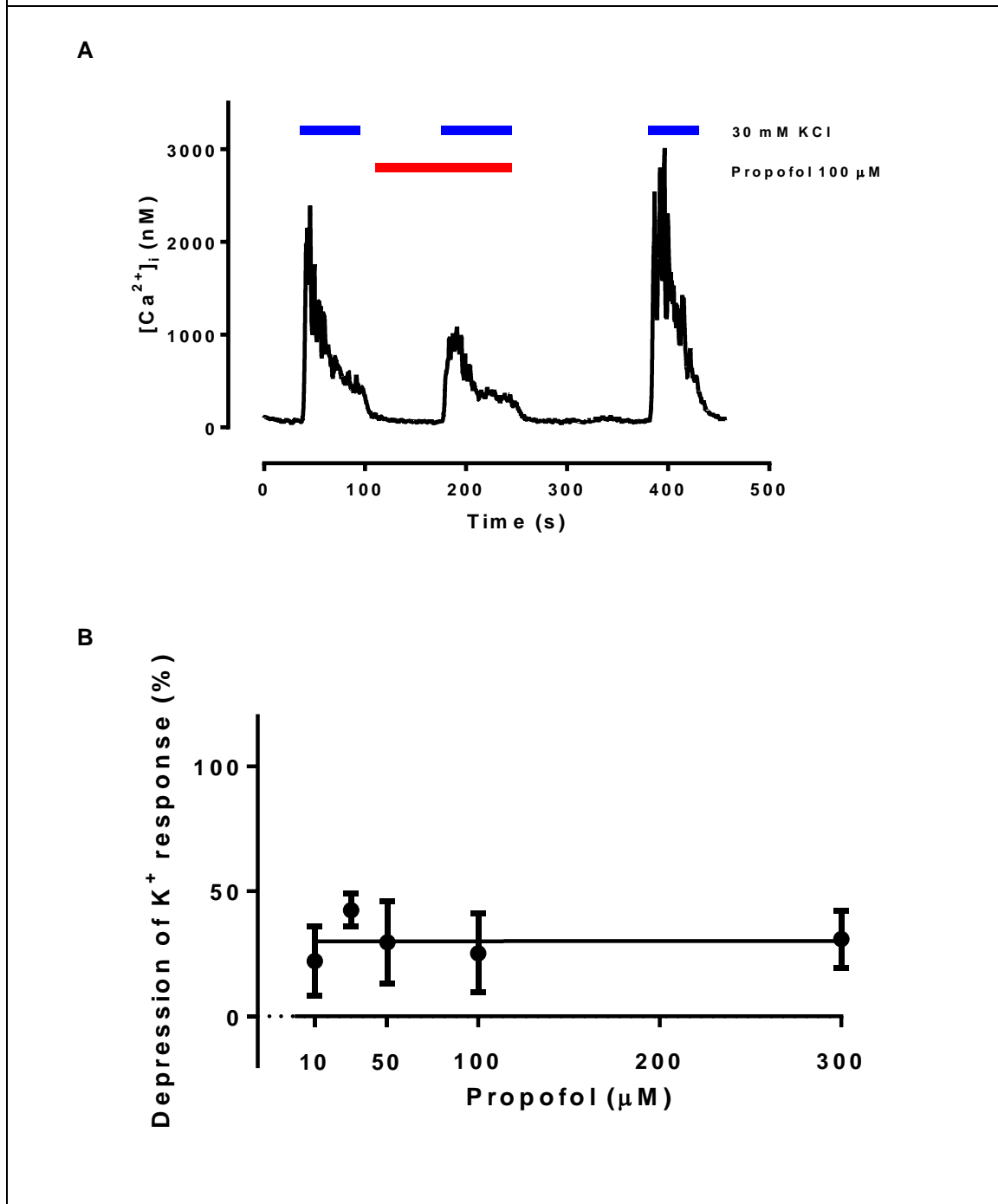
### **Effect of propofol on glomus cell response to high $[K^+]_o$ stimulation**

Again, propofol elicited a small, but significant change in baseline euoxic  $[Ca^{2+}]_i$  in these experiments ( $79 \pm 7$  nM versus  $62 \pm 5$  nM,  $p = 0.002$ ) but modestly blunted the  $[Ca^{2+}]_i$  response to 30 mM KCl (e.g.,  $820 \pm 117$  nM vs  $509 \pm 76$  nM; n=24,  $p < 0.001$ ; at the maximum dose; Figure 4.2). But there was no significant dose-dependent influence of propofol concentration on this depression (ANOVA  $F = 0.371$ ,  $p = 0.775$ ; Figure 4.2B).

**Figure 4.1** Influence of propofol on hypoxia-induced  $[Ca^{2+}]_i$  response in isolated glomus cells. **A.** Example trace of  $[Ca^{2+}]_i$  with exposures to severe hypoxia, showing the near abolition of hypoxic response by 30  $\mu$ M propofol (equivalent to  $\sim 5$   $\mu$ g/ml in human plasma) **B.** Concentration-response relationship of propofol on hypoxic response of glomus cell (magnitude of depression of  $Ca^{2+}$  transient) with propofol dose ( $\mu$ M); mean  $\pm$  SEM ( $n=4-11$  for each data point).



**Figure 4.2** Influence of propofol on  $K^+$ -induced  $[Ca^{2+}]_i$  response in isolated glomus cells. **A.** Example trace of  $[Ca^{2+}]_i$  with exposures to 30 mM KCl, showing some depression of, but not abolition of hypoxic response by 100  $\mu$ M propofol (a dose greater than shown in Figure 4.1). Note the preliminary exposure to hypoxia confirming this is an oxygen-sensitive glomus cell, and the post-exposure control response to KCl that confirms continued cell response to  $K^+$ -induced depolarisation. **B.** concentration-response relationship of  $K^+$ -induced response (magnitude of depression of  $[Ca^{2+}]$  transient) with propofol dose ( $\mu$ M); mean  $\pm$  SEM (n=4-11 for each data point). Drawn to same scale (nM) as **Figure 4.1B** to contrast the differences in magnitude.



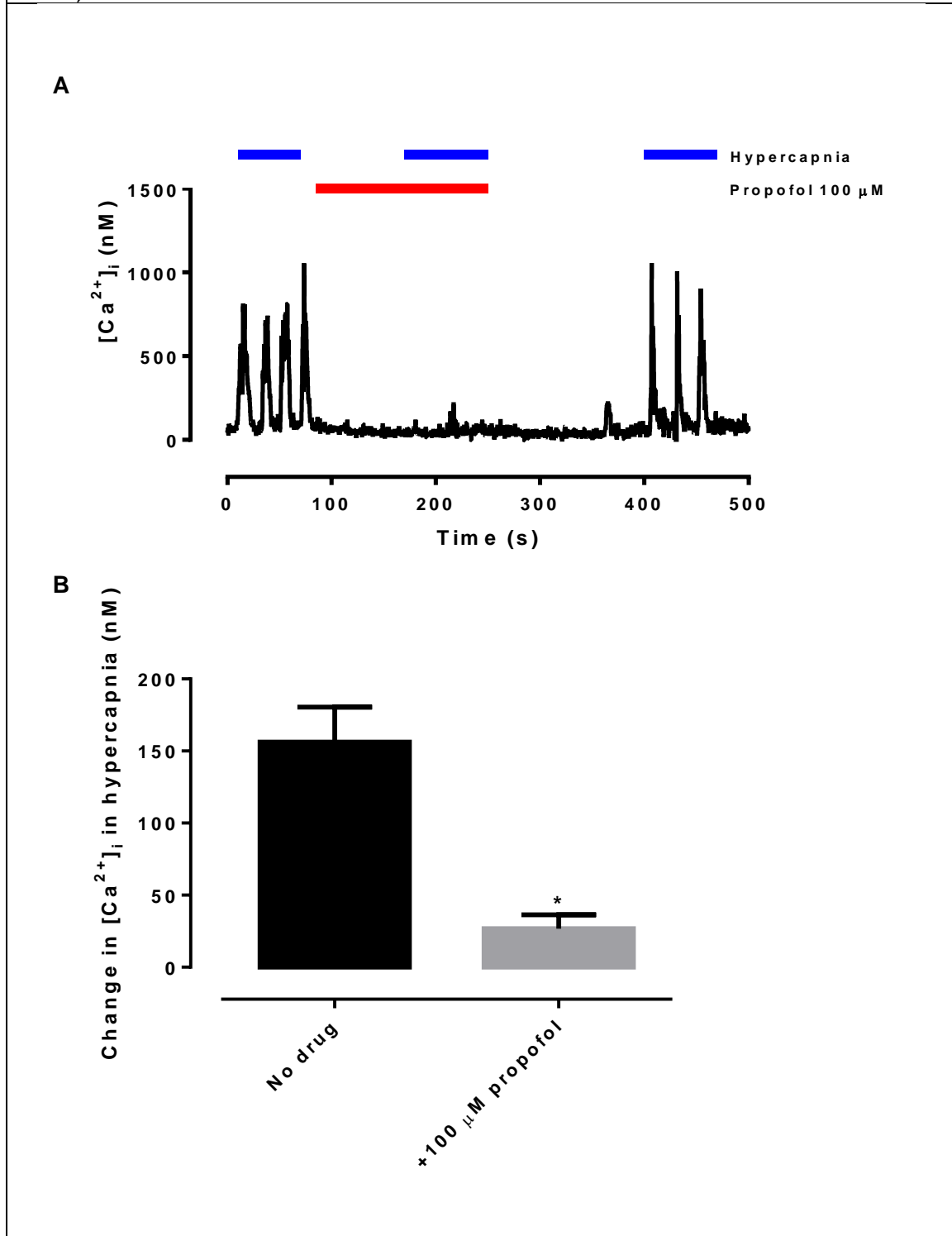
### **Effect of propofol on glomus cell response to CO<sub>2</sub>**

Figure 4.3 shows the effect of propofol on the intracellular [Ca<sup>2+</sup>]<sub>i</sub> response to 20% CO<sub>2</sub>. It is evident that, in the absence of propofol, intracellular [Ca<sup>2+</sup>]<sub>i</sub> shows a repetitive 'spiking' pattern which has been well established as the normal response to this stimulus (Buckler and Vaughan-Jones, 1993; Pandit et al., 2010a). By consistently quantifying the [Ca<sup>2+</sup>]<sub>i</sub> response as an average within the first minute of exposure, when the maximum effect of CO<sub>2</sub> is observed, we avoided the decline observed with sustained hypercapnia (Buckler and Vaughan-Jones, 1994b; Dasso et al., 2000; Pandit et al., 2010a). The effect of propofol was assessed at one dose only, that which had a profound effect on the hypoxic response (Figure 4.1). 100 μM propofol caused a large decrease in the [Ca<sup>2+</sup>]<sub>i</sub> response to 20% CO<sub>2</sub> from 156 ± 24 nM to 27 ± 10 nM (n=7, p=0.003), Figure 4.3.

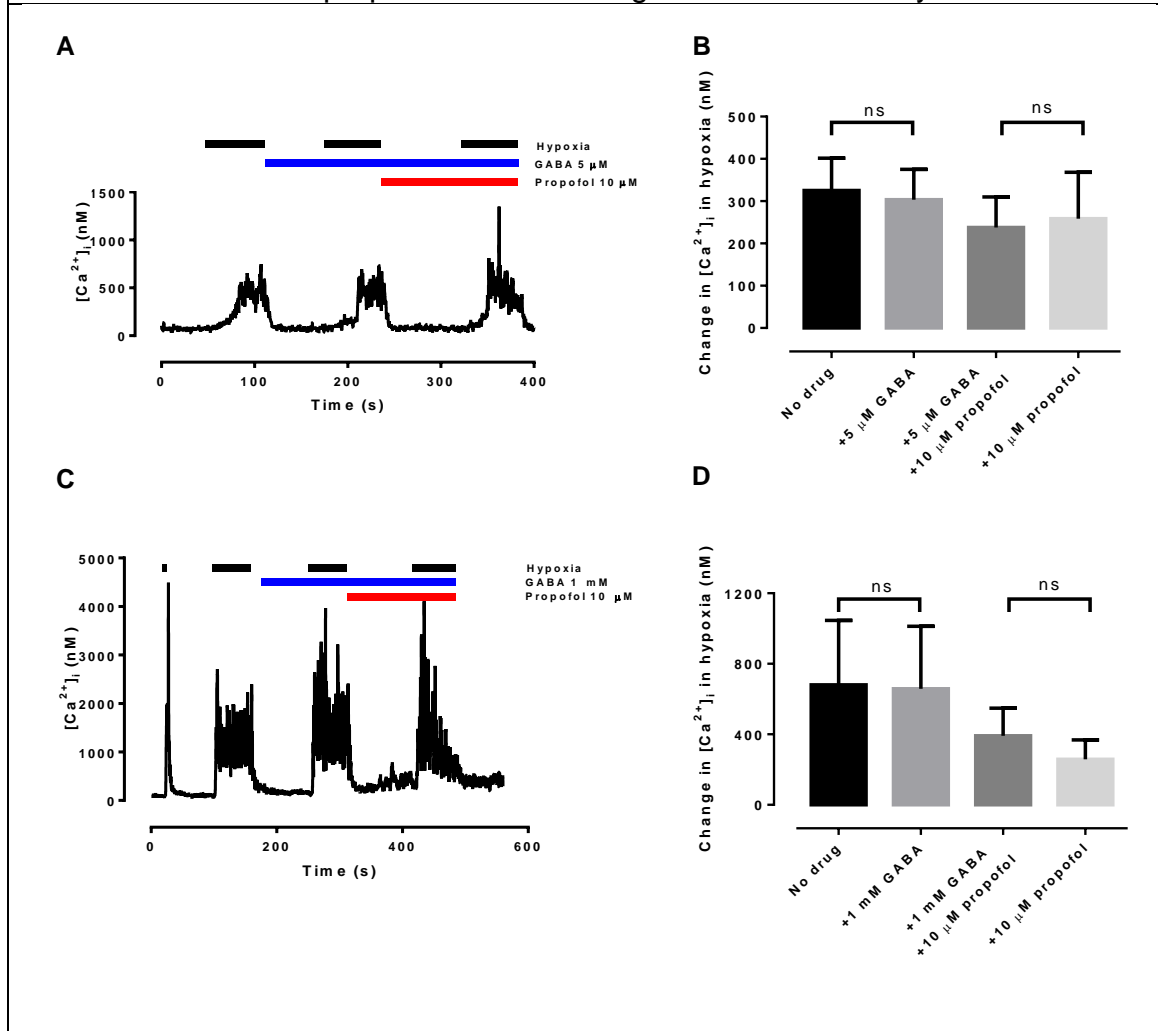
### **Effect of GABA modulation on glomus cell response to hypoxia**

Perfusion of glomus cells with GABA alone across a very wide dose range (5 μM to 1mM; Figure 4.4A and B) did not influence either the baseline euoxic [Ca<sup>2+</sup>]<sub>i</sub> or the [Ca<sup>2+</sup>]<sub>i</sub> response to hypoxia (Figure 4.4; p=0.813 and p=0.136 for 5 μM and 1 mM GABA respectively). Furthermore, propofol at a dose of 10 μM (which alone depressed hypoxic response by 14%, Figure 4.1B) in the presence of either 5 μM or 1 mM GABA had no additional effect (ANOVA, F=0.298, p=0.589; F=0.385, p=0.541 respectively).

**Figure 4.3 A.** Influence of propofol on 20% CO<sub>2</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> response in isolated glomus cells. The cell was first assessed for oxygen sensitivity (note response to severe hypoxia). Then 20% CO<sub>2</sub> was introduced as the stimulus. This response was near-abolished by 100 μM propofol, an effect which was reversible after washout. **B.** Quantitative analysis of the response (mean ± SEM).



**Figure 4.4** Perfusion of glomus cells with GABA alone (5  $\mu$ M or 1 mM) did not influence either the euoxic intracellular  $[Ca^{2+}]_i$  or the  $[Ca^{2+}]_i$  response to hypoxia (**A** and **C**;  $p =$  all ns). Furthermore, the addition of GABA had no additional influence on the effect of propofol at a concentration of 10  $\mu$ M (**B** and **D**). This suggests that GABA has no effect across a wide concentration range and that the effect of propofol on  $O_2$  sensing is not influenced by GABA.



To assess for specific effects of GABA-A vs GABA-B agonism, selective subtype agonists and antagonists were also studied. None of the following had any significant effect on either baseline euoxic  $[Ca^{2+}]_i$  (Table 4.2) or the magnitude of  $[Ca^{2+}]_i$  response to hypoxia (Table 4.3): muscimol (a GABA-A agonist (Krogsgaard-Larsen and Johnston, 1978)) 50  $\mu$ M; baclofen, (a GABA-B agonist (Hill and Bowery, 1981)) 50  $\mu$ M; bicuculline (a GABA-A antagonist

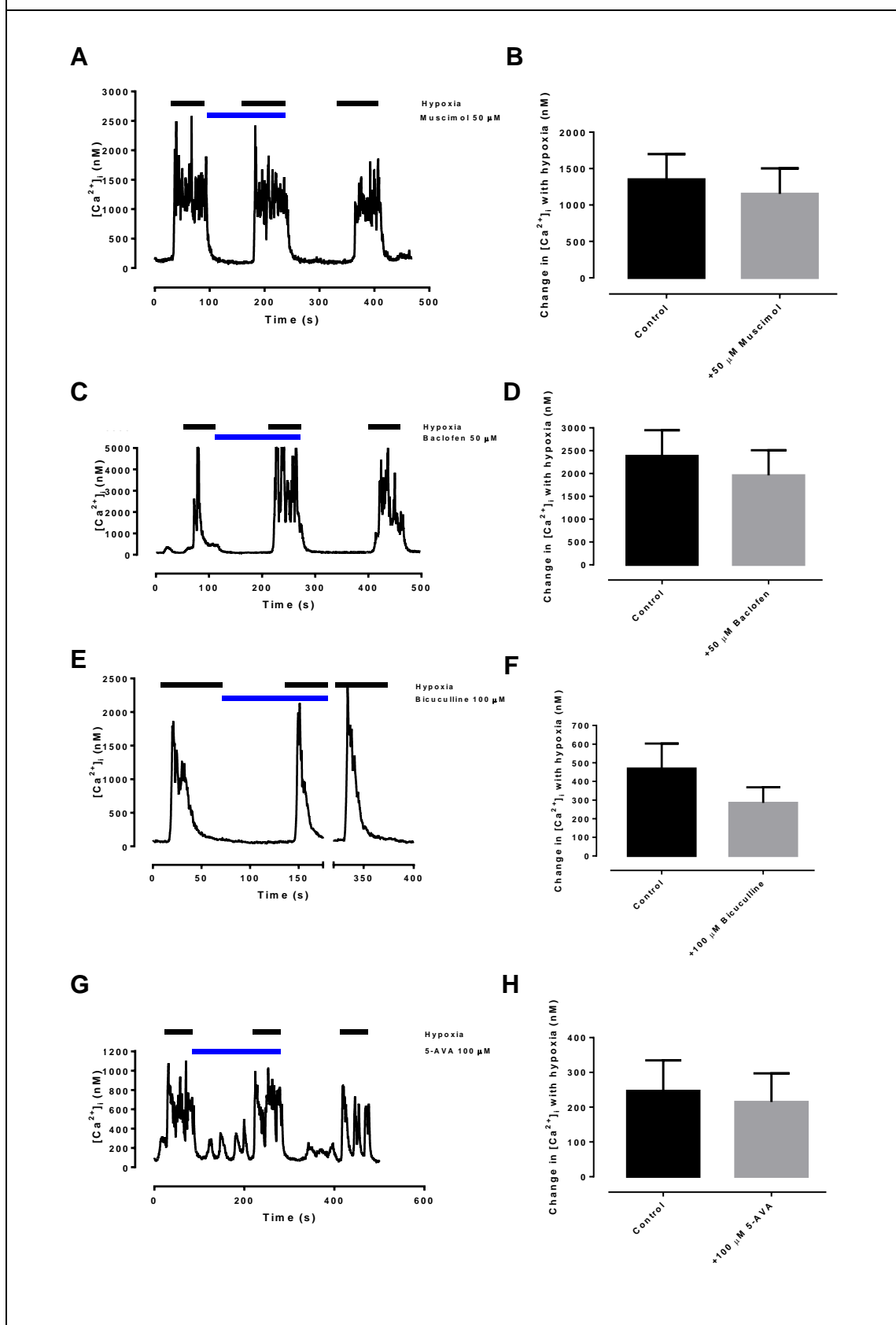
#### *Chapter 4 - Depressive actions of propofol on carotid body glomus cell chemosensation*

(Macdonald et al., 1989)) 100  $\mu$ M; 5-AVA (a GABA-B antagonist (Muhyaddin et al., 1982)) 100  $\mu$ M; Figure 4.5. These were all doses where a measurable effect on the intended target has been demonstrated in similar rat glomus cell preparations (Fearon et al., 2003b; Zhang et al., 2009; Nurse, 2014). Because of this complete lack of effect of either GABA or these specific antagonists/agonists on O<sub>2</sub> sensing, interaction of these agents with added propofol was not further investigated.

#### **Effect of propofol on nicotinic stimulation of glomus cells**

Figure 4.6 shows a representative trace of the effects of nicotine application on [Ca<sup>2+</sup>]<sub>i</sub> and the effect of propofol 100  $\mu$ M in abolishing this response (the quantitative effect shown in Figure 4.6B;  $p < 0.001$ ).

**Figure 4.5 A-B** Muscimol (GABA-A) **C-D** agonising GABA-B using baclofen **E-F** bicuculline (GABA-A antagonism) and **G-H** 5-AVA (GABA-B antagonist). All figures  $[Ca^{2+}]_i$  in nM  $\pm$  SEM.



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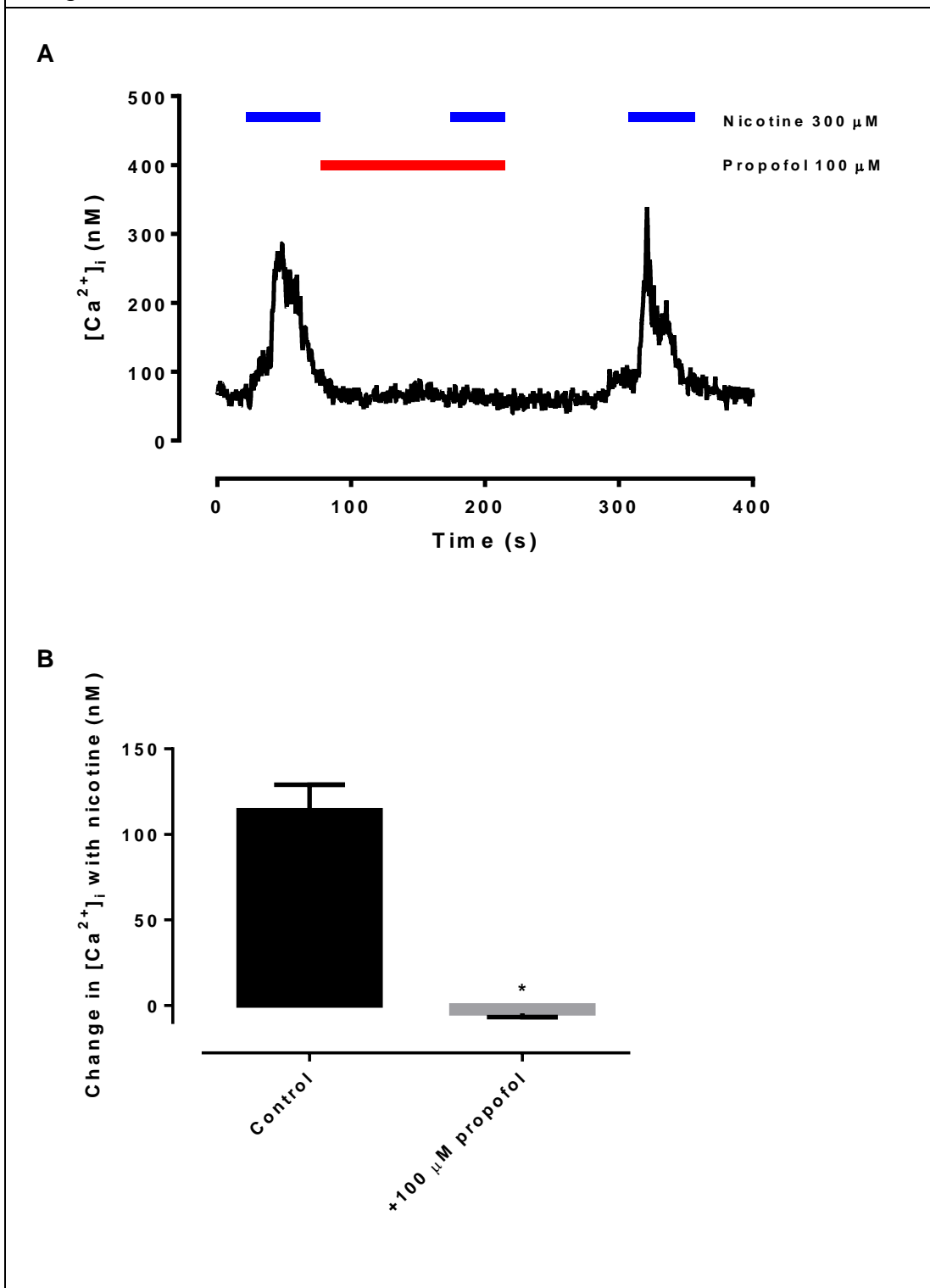
**Table 4.2** Effect of GABA modulators on euoxic  $[Ca^{2+}]_i$ . All figures  $[Ca^{2+}]_i$  in nM  $\pm$  SEM.

	No drug	Drug	n	p
Muscimol	162 $\pm$ 32	128 $\pm$ 20	8	0.129
Baclofen	198 $\pm$ 40	143 $\pm$ 18	7	0.262
Bicuculline	181 $\pm$ 38	165 $\pm$ 37	7	0.721
5-AVA	148 $\pm$ 24	120 $\pm$ 20	17	0.22

**Table 4.3** Effect of GABA modulators on hypoxic increase in  $[Ca^{2+}]_i$ . All figures  $[Ca^{2+}]_i$  in nM  $\pm$  SEM.

	No drug	Drug	n	p
Muscimol	1350 $\pm$ 349	1152 $\pm$ 351	8	0.238
Baclofen	2385 $\pm$ 564	1959 $\pm$ 550	7	0.427
Bicuculline	469 $\pm$ 134	285 $\pm$ 84	7	0.274
5-AVA	247 $\pm$ 88	215 $\pm$ 82	17	0.664

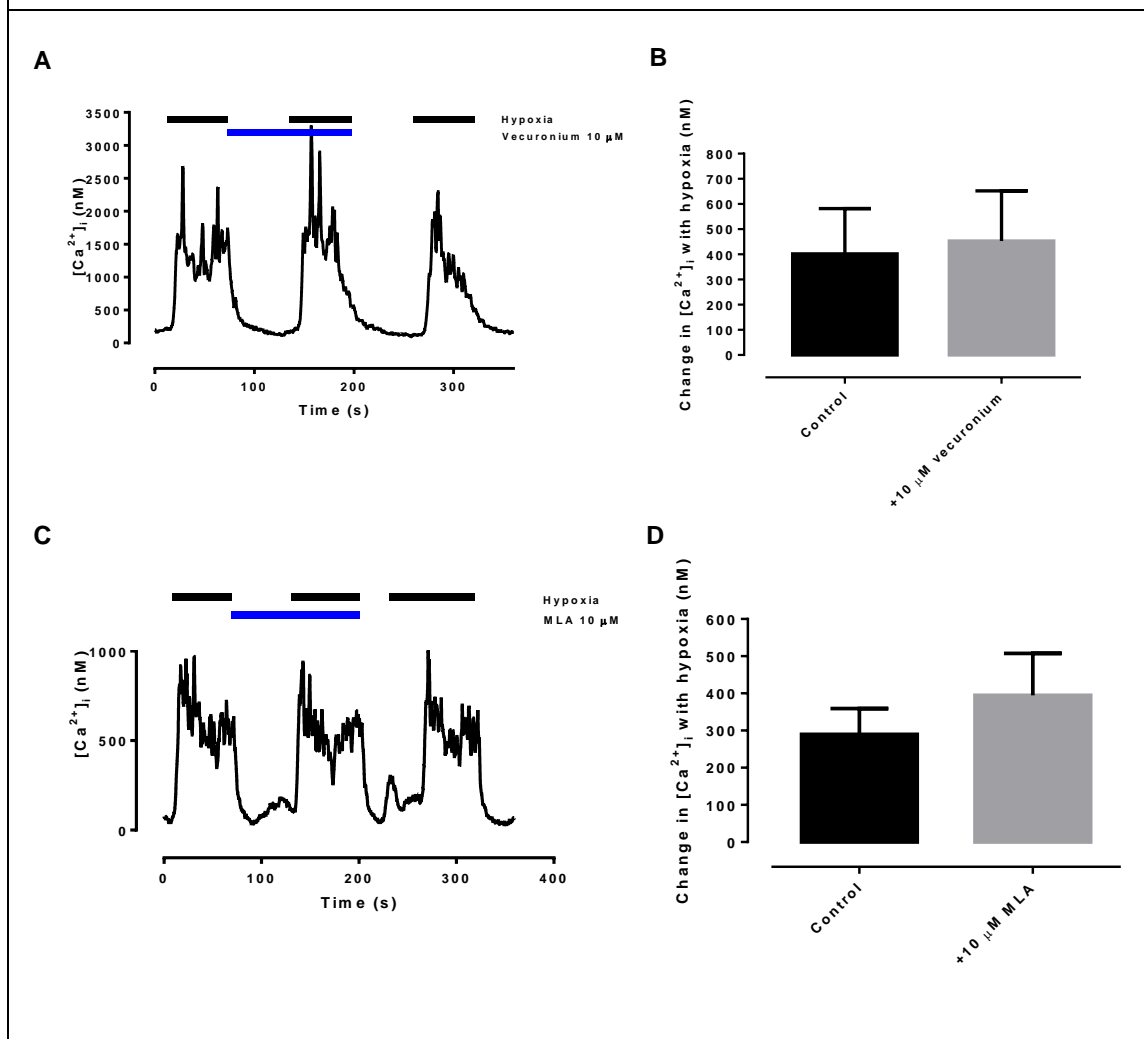
**Figure 4.6 A.** Representative trace of the influence of propofol on nicotine-induced  $[Ca^{2+}]_i$  response in isolated glomus cells. The cell was first assessed for oxygen sensitivity (note response to severe hypoxia). Then nicotine  $300 \mu M$  was introduced as the stimulus. This response was abolished by  $100 \mu M$  propofol; an effect which was reversible after washout. **B.** Quantitative analysis of the response ( $114 \pm 20$  vs  $-5 \pm 2$  nM,  $n=7$ ;  $p < 0.001$ ) All figures mean  $\pm$  SEM.



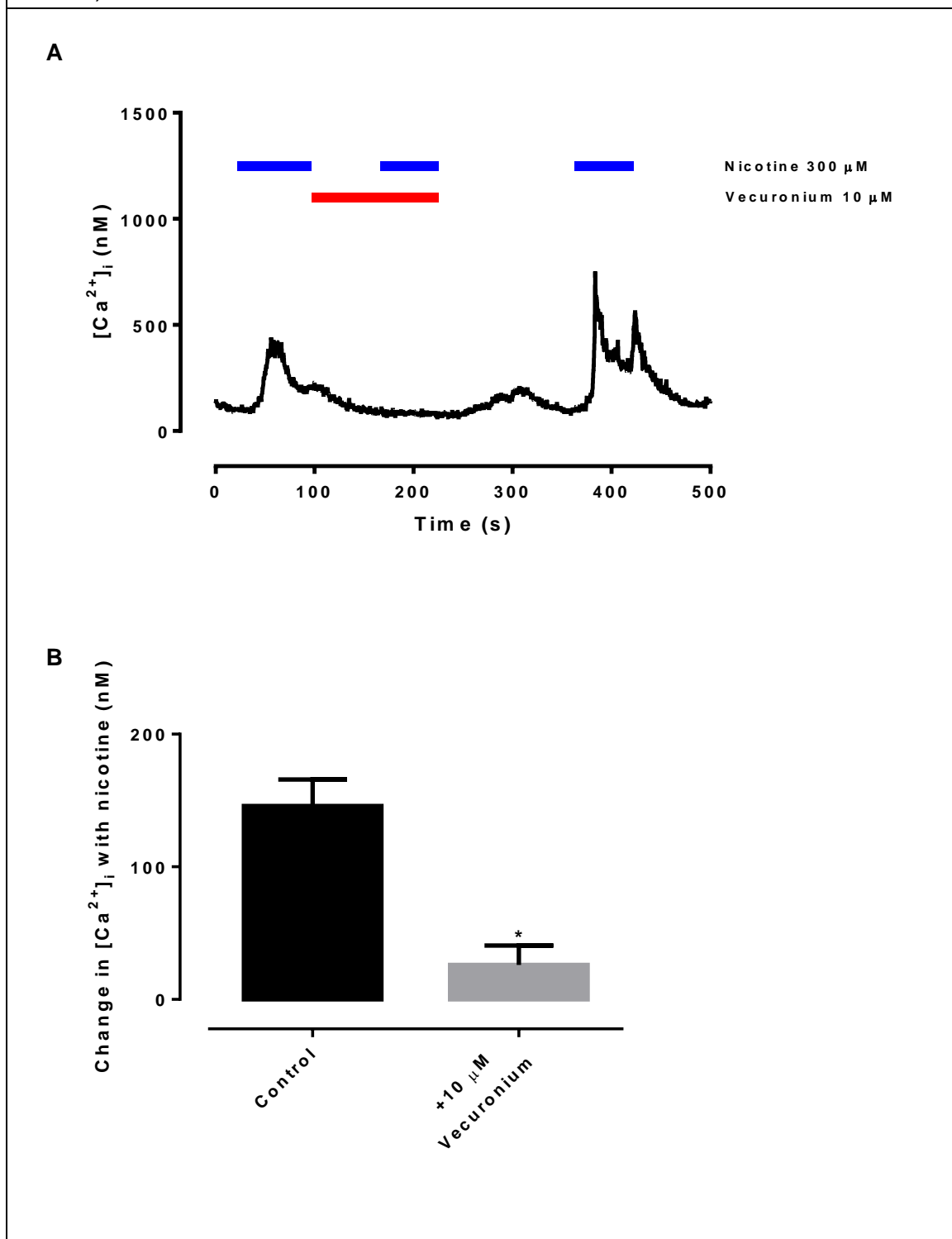
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Neither vecuronium or MLA alone influenced the hypoxia-induced increase in  $[Ca^{2+}]_i$  (Figure 4.7;  $p = 0.807$ ). However, vecuronium did, as might be expected for a nicotinic antagonist, near-abolish the nicotine-induced  $[Ca^{2+}]_i$  response (Figure 4.8;  $p = 0.015$ ).

**Figure 4.7 A-B.** Vecuronium has no effect on hypoxia induced increase in  $[Ca^{2+}]_i$   $402 \pm 180$  vs  $453 \pm 200$  nM,  $p=0.5$ . However, vecuronium may not influence the  $\alpha 7$  homomeric nAChR. To ensure pharmacological blockade of this isoform, MLA was applied. **C-D.** This again showed no effect on hypoxia induced increase in  $[Ca^{2+}]_i$  ( $290 \pm 69$  vs  $395 \pm 113$  nM,  $p=0.367$ ) All figures mean  $\pm$  SEM.



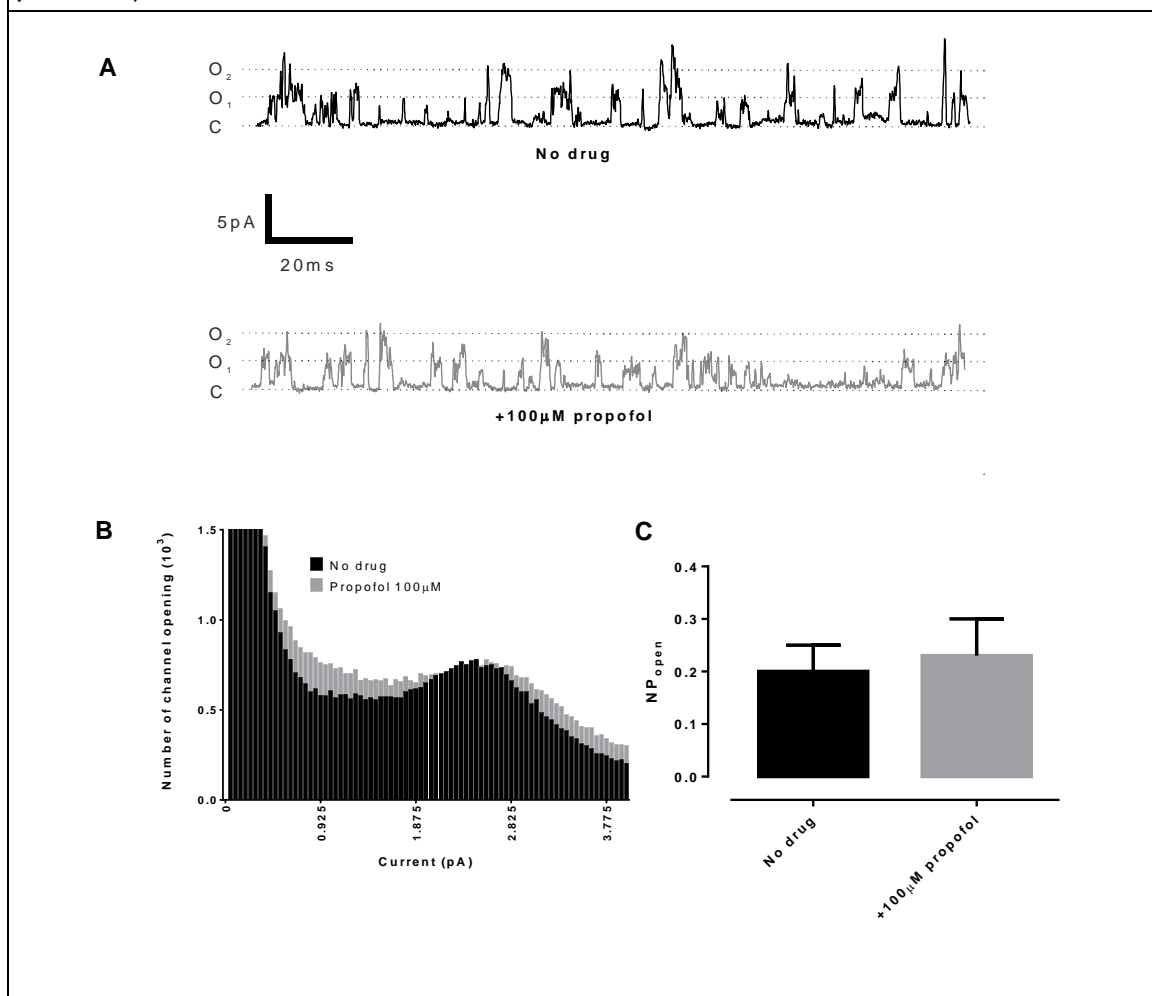
**Figure 4.8 A.** Representative trace of vecuronium influence on nicotine-induced rise in  $[Ca^{2+}]_i$  in isolated glomus cells. Note the prior brief exposure to mild hypoxia, the initial rise in  $[Ca^{2+}]_i$  with nicotine, its near-abolition by vecuronium, and restoration of both nicotine- and hypoxia-induced  $[Ca^{2+}]_i$  response after washout. **B.** Quantitative analysis ( $146 \pm 20$  vs  $26 \pm 15$  nM, mean  $\pm$  SEM;  $n = 4$ ,  $p = 0.015$ ).



### Effect of propofol on TASK channels

There was no significant effect of propofol 100  $\mu$ M on TASK channel open probability (0.19 vs 0.22,  $n=11$ ,  $p=0.088$ ). Figure 4.9 shows the characteristic all-points histogram of the native TASK current, with an example trace above. Overlaid is the histogram after the application of propofol, and it can be clearly seen that there was no change at the peak corresponding to TASK at  $\sim 2.5$  pA (Figure 4.9C).

**Figure 4.9** TASK electrophysiology. An all-points histogram **B** is used to define the characteristic peak current ( $\sim 2.5$  pA) in each patch in each condition (**A** example trace of single channel openings: grey with propofol 100  $\mu$ M, black without) using the cell as its own control. **C**. Quantitative analysis of the  $NP_{open}$  (the probability of a single channel opening event) showed no significant influence of propofol on channel opening (0.19 vs 0.22,  $n=11$ , mean  $\pm$  SEM,  $p=0.088$ ).



### **Discussion**

The main finding of this chapter is that propofol does exert a direct inhibitory effect on the glomus cell response to hypoxia and CO<sub>2</sub>, obtunding the rise in [Ca<sup>2+</sup>]<sub>i</sub> evoked by these stimuli. This would be entirely consistent with observations of its chemodepressant effects in human volunteers and isolated perfused carotid body preparations.

I have confirmed that nicotine also stimulates a rise in [Ca<sup>2+</sup>]<sub>i</sub>, indicating as previous work has done that cholinergic mechanisms may be important presynaptically within the carotid body (Wyatt and Peers, 1993; Dasso et al., 1997). However, the cholinergic antagonist vecuronium obtunded only the nicotine-induced, but not hypoxia-induced rise in [Ca<sup>2+</sup>]<sub>i</sub> (Figure 4.6 and Figure 4.7). This is a novel result, which suggests that nicotinic mechanisms may not, in fact, be integral to the glomus cell hypoxic response, but play a role in autocrine, paracrine and neural afferent signalling once hypoxic activation and neurosecretion has already occurred, consistent with the work of Chen et al. (1999). If this is the case, my results suggest that while propofol undoubtedly blocks a nicotineric activation mechanism (Figure 4.6), it does not act via such a mechanism to inhibit the *hypoxia-induced* activation of glomus cells. The action of propofol in respect of hypoxic reflex inhibition must therefore be by some novel mechanism.

In the light of these novel results, the previous work of Jonsson et al. (2005) requires some re-interpretation. Given their results, it was appropriate to conclude that any inhibitory effect of propofol on hypoxic sensing in an isolated perfused carotid body-sinus nerve preparation might be exerted at the level of

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the postsynaptic membrane: hypoxia is thought to induce ACh secretion from glomus cells (Wyatt and Peers, 1993; Dasso et al., 1997; Shirahata et al., 2007), which acts at postsynaptic nicotinic receptors (Tassonyi et al., 2002), these receptors being presumably directly inhibited by propofol. However, if propofol suppresses the hypoxia-induced rise in  $[Ca^{2+}]_i$  to the degree I discovered (Figure 4.1), then there is unlikely to be significant neurosecretion in the first place. This does not alter the importance of the Jonsson et al. (2005) results, especially their relevance for clinical practice. They reported, for example, that cholinergic antagonists (i.e., the neuromuscular blocker vecuronium) inhibit hypoxic response in isolated carotid body preparations. Since I found that vecuronium has no effect on oxygen sensitivity at carotid body level, these important effects of neuromuscular blockade must arise at cell to cell level as Jonsson et al. (2005) correctly suggested. Thus, the effects of propofol and residual neuromuscular blockade could synergise in the postoperative period by a range of mechanisms to profoundly depress the hypoxic and/or hypercapnic ventilatory response. The result that propofol does not influence the function of TASK channels (Figure 4.9) is consistent with results obtained in TASK channels expressed in oocytes (Putzke et al., 2007), and extends that work to the native channels. Furthermore, it suggests that propofol acts in a manner to depress hypoxia- and CO<sub>2</sub>-sensing distinct from volatile anaesthetics, where there is growing evidence that these (halothane and isoflurane particularly) increase open probability of TASK channels (Patel et al., 1999; Sirois et al., 2000; Shin and Winegar, 2003; Pandit et al., 2010b).

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Since many actions of propofol, such as hypnotic, are thought to occur via GABA receptors (Houston et al., 2012; Franks, 2015), I might have expected its depressive effect on hypoxia-evoked  $[Ca^{2+}]_i$  also to be influenced by exogenously applied GABA and GABA agonists, especially as there are known to be GABA receptors on the glomus cell membrane. The fact that I did not see this excludes GABA mediation as responsible for propofol's chemodepressant actions.

Fearon et al. (2003b) have argued that GABA acts in a negative feedback manner to modulate TASK channel activity: the theory is that the GABA released from glomus cells into synaptic cleft acts postsynaptically, but also presynaptically to prevent further GABA release. However, I observed no significant effects of GABA, GABA agonists or antagonists, or any interaction of GABA with propofol (Figure 4.4 and Figure 4.5). These results would be consistent with the hypothesis that the primary role of GABA is postsynaptic, as suggested by Zhang et al. (2009), and that any presynaptic modulatory action is weak or secondary.

In addition to its action on nicotinic mechanisms, it was noted that propofol had a modest depressive action on voltage-gated calcium ( $Ca^{2+}_v$ ) entry into glomus cells, possibly acting via voltage-gated  $Ca^{2+}$  channels (Figure 4.2). In a variety of models with an array of channel subtypes,  $Ca^{2+}_v$  has been invulnerable to propofol (Hall et al., 1994; Orestes and Todorovic, 2010). A notable exception is the slow inactivating T-type  $Ca^{2+}_v$  channel, which is blocked by propofol (Joksovic et al., 2005). Recent work has proposed a role for the T-type channel in carotid body chemosensing (Makarenko et al., 2015). However, in my

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preparation this depressive effect was not concentration-dependent, making this  $\text{Ca}^{2+}_v$  channels less likely to be the sole means by which propofol inhibits hypoxic- and  $\text{CO}_2$ -sensing.

Nevertheless it is clear that, taking all my results and those of other groups together, like so many anaesthetics propofol has diverse actions: depression of nicotinic transmission in the glomus cell; postsynaptic depression of nicotinic transmission; and possible (albeit limited) depression of voltage-gated  $\text{Ca}^{2+}$  entry. I did not explore the possibility of phosphorylation-mediated channel regulation (e.g. through  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II, CaMKII). This is a potential explanation for the polymodal depression of chemosensation as propofol has been demonstrated to reduce CaMKII phosphorylation (Cui et al., 2009), a potent mediator of multiple pertinent ion channels (e.g. nAChR (Ridley et al., 2001)). Neither can we exclude that cellular calcium homeostasis is affected by propofol, for instance through an effect on calcium storage, buffering or active transport.

One limitation of the current study is that I do not know if propofol acts directly on nicotinic receptors on glomus cells to achieve its depressive effects on hypoxia- and  $\text{CO}_2$ -sensing, or if its action is indirect; i.e., that nicotinic receptors are instead modulated by other factors that are influenced by propofol. Evidence for the former is that propofol has a direct effect on the nicotinic receptor in excised patches of cancer cells (Dilger et al., 1994) and in channel expression systems (Flood et al., 1997; Violet et al., 1997).

To explore these potential channel effects further, a comprehensive electrophysiological analysis of the effect of propofol on  $\text{Ca}^{2+}_v$  and nAChR in

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glomus cell membranes (in the excised patch configuration) would be required, which is beyond the scope of this study.

I did not assess the role of propofol across a range of chemostimuli strengths. This could potentially reveal a different effect of propofol at “milder” levels of hypoxia, as recent evidence suggests recruitment of a novel sodium conductance only in severe hypoxia (Kang et al., 2014). It is also of note that similar to the work of Jonsson et al. (2005) and Akada et al. (2008), I applied concentrations of propofol in the range of 10-500  $\mu\text{M}$ . Though it makes this study directly comparable with other in vitro work, as propofol is highly protein bound (Servin et al., 1988) the free plasma concentration of propofol is likely to be lower in clinical practice.

In summary I have demonstrated that:

- Propofol depresses hypoxia- and hypercapnia-induced  $\text{Ca}^{2+}$  influx into glomus cells.
- This appears to be via a novel mechanism, independent of nicotinic processes, and TASK or GABA channel functions.

**Chapter 5:**

**THE INFLUENCE OF DEXMEDETOMIDINE ON GLOMUS  
CELL AND RODENT RESPONSE TO HYPOXIA**

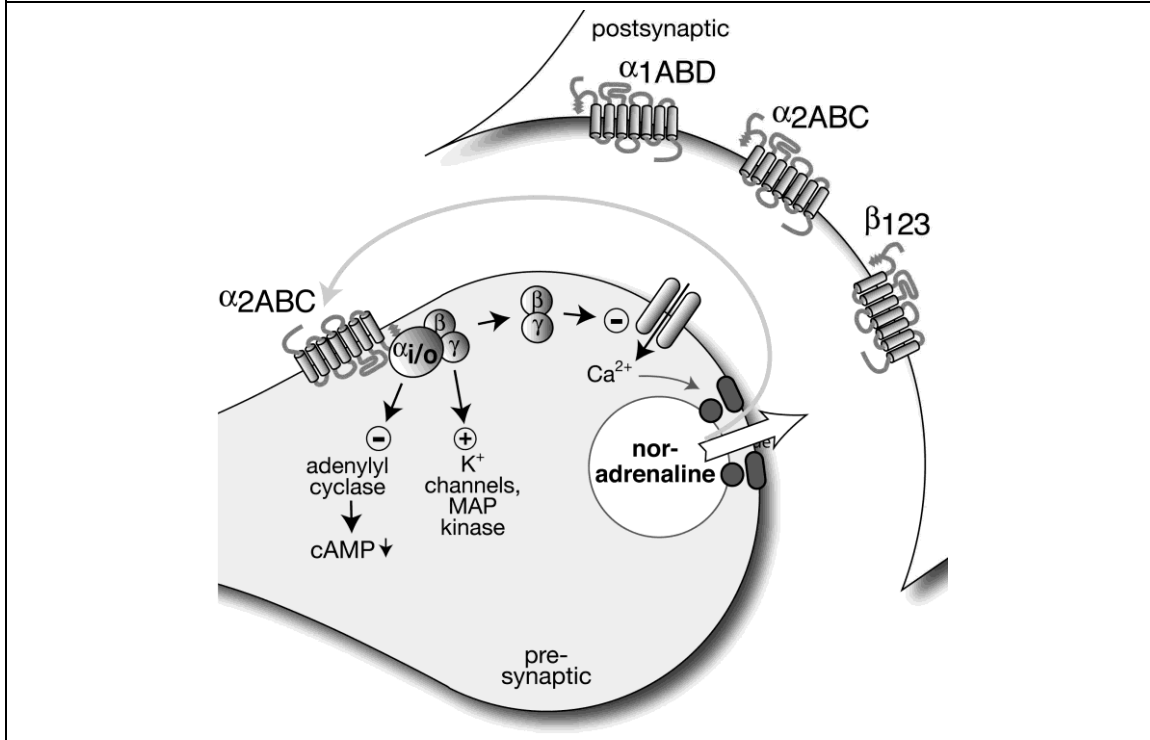
### **Overview**

The  $\alpha_2$ -adrenoceptors are expressed pre-synaptically in many tissue types and species. Their role is predominantly thought of as regulating excitatory neurotransmission through presynaptic negative feedback. Through their coupling to Gi-proteins, they inhibit the production of cAMP from ATP. The resultant decrease in cAMP-dependant protein kinase activity has a myriad of intracellular effects, including lipolysis (Wright and Simpson, 1981), glycogenolysis (DiTullio et al., 1984) and regulation of smooth muscle and vascular tone (Langer et al., 1985).

In the context of synaptic transmission, the endogenous agonists of  $\alpha_2$ -adrenoceptors are noradrenaline and adrenaline, the former having the higher affinity. The presence of these receptors on the presynaptic terminal of neurones is key in the autoinhibitory feedback mechanism (Figure 5.1). Noradrenaline release, following stimulatory neurosecretion, results in a decrease in further noradrenaline release through a pre-synaptic  $\alpha_2$ -adrenoceptor, cAMP dependant inhibition of calcium channels. This damping of noradrenaline release can be of up to 90% in some tissues (Rang et al., 1999), implying that perturbing this autoregulation could have a significant physiological consequence in excitable tissues.

**Figure 5.1** Representation of synaptic role of adrenoceptors. Noradrenaline binds to pre-synaptic  $\alpha_2$ -adrenoceptors to effect a decrease in cAMP and ultimately reduce calcium influx into cells.

Picture reproduced from Gilsbach and Hein (2008). "Schema eines Autorezeptors" by Professor Lutz Hein is licensed under CC BY-SA 3.0 DE



Recent work has suggested that acutely catecholamines are inhibitory of carotid sinus nerve discharge in rats (Hauton et al., 2013). However, in the same study, it was postulated that the effects of catecholamines in vivo may be multiple and complex, with an array of adrenoceptor ( $\alpha$  and  $\beta$ ) effects converging to cause an *augmentation* of ventilation.

$\alpha_2$ -adrenoceptors have been demonstrated by radio-binding assays on rabbit glomus cells (Kou et al., 1991). In the intact carotid body, these cells represent a pre-synaptic site: when excited by hypoxia, neurosecretion acts to stimulate post synaptic receptors of the carotid sinus nerve. So, an autoinhibitory feedback mechanism plausibly operates within the carotid body as a

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mechanism to limit or regulate the amount of neurotransmitter released in response to hypoxic or hypercapnic stimulus.

There has long been interest in  $\alpha_2$ -adrenoceptor modulation as a therapeutic target, particularly in the treatment of hypertension. In veterinary practice, medetomidine and xylazine have been used as a sedative-analgesic widely for many decades. Clonidine is in widespread clinical use in humans, though it is only a partial agonist at the  $\alpha_2$ -adrenoceptor. Dexmedetomidine is a non-selective alpha2-adrenoceptor ( $\alpha_2$ ) agonist (Paris and Tonner, 2005), and a full agonist for the  $\alpha_{2b}$  receptor subtype (Peltonen et al., 1998), which has been in clinical use worldwide since 2010.

Since the initial descriptions of dexmedetomidine (Maze and Tranquilli, 1991; Belleville et al., 1992; Dyck et al., 1993), there has been considerable debate over its effects on respiratory control. Some reports suggesting little or no depressive effect on hypoxic/hypercapnic responses (Nguyen et al., 1992; Nakatani et al., 2012). However, in rabbits and rats respectively, Nishida et al. (2002) and Fernandes et al. (2006) reported that dexmedetomidine was depressive of breathing control. The latter would be in common with the respiratory chemoreflex depression described for many other anaesthetics (van den Elsen et al., 1998; Pandit et al., 1999a; Nishida et al., 2002; Pandit, 2002; Pandit and Moreau, 2005; Fernandes et al., 2006; Pandit and O'Gallagher, 2008).

One limitation of the Nishida et al. (2002) study was that the animals continued to inhale up to 0.3% sevoflurane whilst dexmedetomidine was infused, which at these concentrations itself can modestly reduce the ventilatory response for

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hypoxia (Sarton et al., 1996; Pandit et al., 1999b) - but not for CO<sub>2</sub> (van den Elsen et al., 1998; Pandit, 2005).

Similarly, in an infusion and bolus regimen sufficient to cause significant sedation, a limited report suggests that dexmedetomidine impaired both hypoxic and hypercapnic ventilatory responses in a volunteer study (Danielson et al., 2014), equivalent to propofol. In contrast, Nguyen et al. (1992) reported in dogs that high dose (up to 100 mcg/kg) dexmedetomidine stimulated ventilation and chemoreflex responses.

More recently, Nakatani et al. (2012) reported that carotid sinus nerve activity from excised carotid bodies (rabbits) increased in response to hypoxia when perfused with low dose dexmedetomidine. Nakatani et al. (2012) used a reductive approach to the problem, by studying more directly the influence of the drug on the acute hypoxic ventilatory response (AHVR) by measuring carotid sinus nerve afferent activity in isolated, perfused rabbit carotid bodies.

Hypoxia causes membrane depolarisation through closure of leak potassium channels and results in an increased intracellular calcium ( $[Ca^{2+}]_i$ ) and subsequent neurotransmitter release to activate the carotid sinus nerve and initiate autocrine/paracrine signalling pathways (Kumar and Prabhakar, 2011; Nurse, 2014). Thus the findings of Nakatani et al. (2012) and Nguyen et al. (1992) imply not only that dexmedetomidine had no effect on response but that the glomus cells were *stimulated* by dexmedetomidine.

Sedative or anaesthetic (volatile and intravenous) drugs generally depress the protective chemoreflex increase in ventilation in the face of hypoxaemia or

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hypercapnia (Knill and Gelb, 1978; Temp et al., 1994; Nagyova et al., 1995; Pandit et al., 1999a; Pandit, 2002), perhaps the latter being somewhat better preserved than the former (Pandit et al., 1999b; Pandit, 2005; Pandit and Moreau, 2005).

The answer to this question is of key mechanistic importance, as the excitability of these cells (and the function of their background TASK-like channels) has been shown to be depressed by a variety of inhalational anaesthetics (Buckler et al., 2000; Pandit and O’Gallagher, 2008; Pandit and Buckler, 2009; Pandit et al., 2010b) and similarly depressed by intravenous agents (Ponte and Sadler, 1989a; Jonsson et al., 2005; Akada et al., 2008): consistent with the effects of all these drugs at whole body level.

Yet, little is currently understood about how dexmedetomidine might act on the glomus cell. The possibility that there exists a hypnotic drug devoid of these adverse depressive actions on the chemoreflex would therefore be of considerable interest.

In light of these previous studies, I wished to determine whether dexmedetomidine augments the hypoxia-induced calcium influx into carotid body glomus cells (consistent with the results of Nguyen et al. (1992) and Nakatani et al. (2012)), or if it has no effect (consistent with the findings of Nishida et al. (2002) and Fernandes et al. (2006)). Furthermore, I wished to investigate whether any effects that were observed at cellular level were consistent with the effects of dexmedetomidine on whole-body chemoreflexes in intact animals.

I therefore hypothesised that:

- (1) Dexmedetomidine would augment the hypoxia induced  $[Ca^{2+}]_i$  response in glomus cells at low concentrations.
- (2) Low dose dexmedetomidine would augment the AHVR of freely moving mice.
- (3) Higher doses of dexmedetomidine would have a neutral or depressive effect on the AHVR of mice, as the sedative effect becomes apparent.

### **Methods**

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986. Neonatal Sprague-Dawley rats (P11-14) were used for the isolated glomus cell study and C57BL6 inbred mice (7-8 weeks old) were used for the whole-animal plethysmography study. Both species were supplied by Harlan (Blackthorn, Oxfordshire).

Carotid bifurcations were dissected from rat pups, as described in Chapter 2 General Methods. In brief, the excised carotid bodies were subjected to enzymatic treatment and triturated to isolate individual cells in suspension as previously described (Buckler and Vaughan-Jones, 1993; Buckler et al., 2000). Cells were loaded with indo-1, AM dye for 60 minutes. Intracellular calcium concentration was estimated in these cells using ratiometric fluorimetry. Each cell was sequentially superfused with 37°C Tyrode's solution equilibrated with 5% CO<sub>2</sub> in balanced air (euoxia) for 5 - 10 min and 5% CO<sub>2</sub> with 1% O<sub>2</sub> in balanced N<sub>2</sub> (mild hypoxia, 15 mm Hg O<sub>2</sub>) or 5% CO<sub>2</sub> in balanced N<sub>2</sub> (severe hypoxia, 0% O<sub>2</sub>, <3 mm Hg O<sub>2</sub>) for periods of at least one minute and up to 3 min each, returning to euoxia each time for between 5 – 10 min. All cells were exposed to

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both control and drug so that each acted as its own control. On any given day, only one severity of hypoxic stimulus was used, but the order of drug doses were randomised by coin toss.

This sequence was repeated with the addition of dexmedetomidine to the superfusate in the concentrations 0.1 nM and 1 nM. The change in intracellular concentration ( $[Ca^{2+}]_i$ , nM) in response to hypoxia was calculated as the average value over the period of hypoxia, subtracted from the mean of  $[Ca^{2+}]_i$  values in the 30 sec euoxic period before the switch into hypoxia, and compared for each cell in the presence and absence of dexmedetomidine.

Whole body plethysmography was performed by placing conscious mice in plexigas chambers (Buxco Electronics, Wilmington, NC), which were continually flushed with gas mixtures at a flow rate of 2 l.min<sup>-1</sup>. Pressure changes were measured with a differential pressure transducer, the signal amplified, filtered and converted from analogue-to-digital through a preamplifier. Signal processing was performed by Finepointe software (Buxco Electronics, Wilmington, NC). Oxygen and carbon dioxide concentration within the chamber were measured by continuous sampling from a side-port (Datex Capnomac Ultima, Finland). Animals were acclimatised to the chambers for a period of 30 minutes prior to experimentation.

All experiments were conducted at room temperature, between 0800 and 1400. Hypoxic stimulus was performed with two 5-minute step changes from air into 10% O<sub>2</sub>, 3% CO<sub>2</sub>, 87% N<sub>2</sub> (isocapnic hypoxia) with periods of euoxia in between. The AHVR was calculated as the difference between the peak minute ventilation achieved within 90 seconds after the switch into hypoxia (allowing

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30 seconds for the gas concentration in the chamber to equilibrate) and the mean resting minute ventilation over 90 seconds before the gas switch. The increase in minute ventilation was indexed to (i.e., divided by) the resting minute ventilation to give AHVR as a percentage increase from baseline, to account for variations in absolute levels of resting breathing between mice. Mice were randomised in groups of 4 to receive either dexmedetomidine subcutaneously (5 µg/kg, 50 µg/kg or 500 µg/kg) or vehicle (equivalent volume NaCl 0.9%) in age-matched control animals. These doses are less than those used in veterinary practice as co-anaesthetic (e.g. 1 mg/kg (Gargiulo et al., 2012)), at the lowest dose I would expect no demonstrable analgesic or sedative effect, and at 50-500 µg/kg subtle motor deficits or hypnosis may be detectable (Hunter et al., 1997; Linden et al., 2006). All animals compared were kept in identical housing conditions. Thus on any given day, 4 mice received either drug or saline control with each group of 4 mice receiving only a single dose of drug. There was no significant interaction between day and AVHR ( $p=0.855$ ).

I conducted a power analysis for the animal experiments. Based on the variation in minute ventilation observed during preliminary experiments (see Supplementary Material) with C57BL6 animals (variance  $1 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ ) a power calculation ( $\alpha=5\%$ ,  $\beta=80\%$ ) to detect a  $2 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$  change in AHVR estimated an appropriate sample size of 4 animals in each group.

Factorial analysis of variance was used to assess the influence of different doses of dexmedetomidine on the ventilatory endpoints for both isolated carotid body and whole animal studies (IBM-SPSS version 20.0, IBM-SPSS Science Inc., Chicago, IL). For the former, the end-point was  $[\text{Ca}^{2+}]_i$  and there were the

following factors: hypoxia (two levels, one for each stimulus level, mild or severe) and dexmedetomidine (two levels, one for each dose). For the latter, the end-point was minute ventilation and there was one factor, dexmedetomidine (three levels, one for each dose). Where significance was suggested post-hoc Bonferroni tests were applied.

## **Results**

### **Glomus cell calcium measurement in hypoxia**

Figure 5.2 shows a typical glomus cell  $[Ca^{2+}]_i$  recording of mild hypoxia, with and without dexmedetomidine. With milder levels of hypoxia, I noticed a more 'spiky'  $[Ca^{2+}]_i$  response (as seen in Figure 5.2A), whereas the more robust response elicited with severe hypoxia generally yielded a more stable  $[Ca^{2+}]_i$  response pattern, consistent with previous reports (Buckler and Vaughan-Jones, 1994; Dasso et al., 2000). The method of calculating glomus cells hypoxic response thus took into account this range of response types.

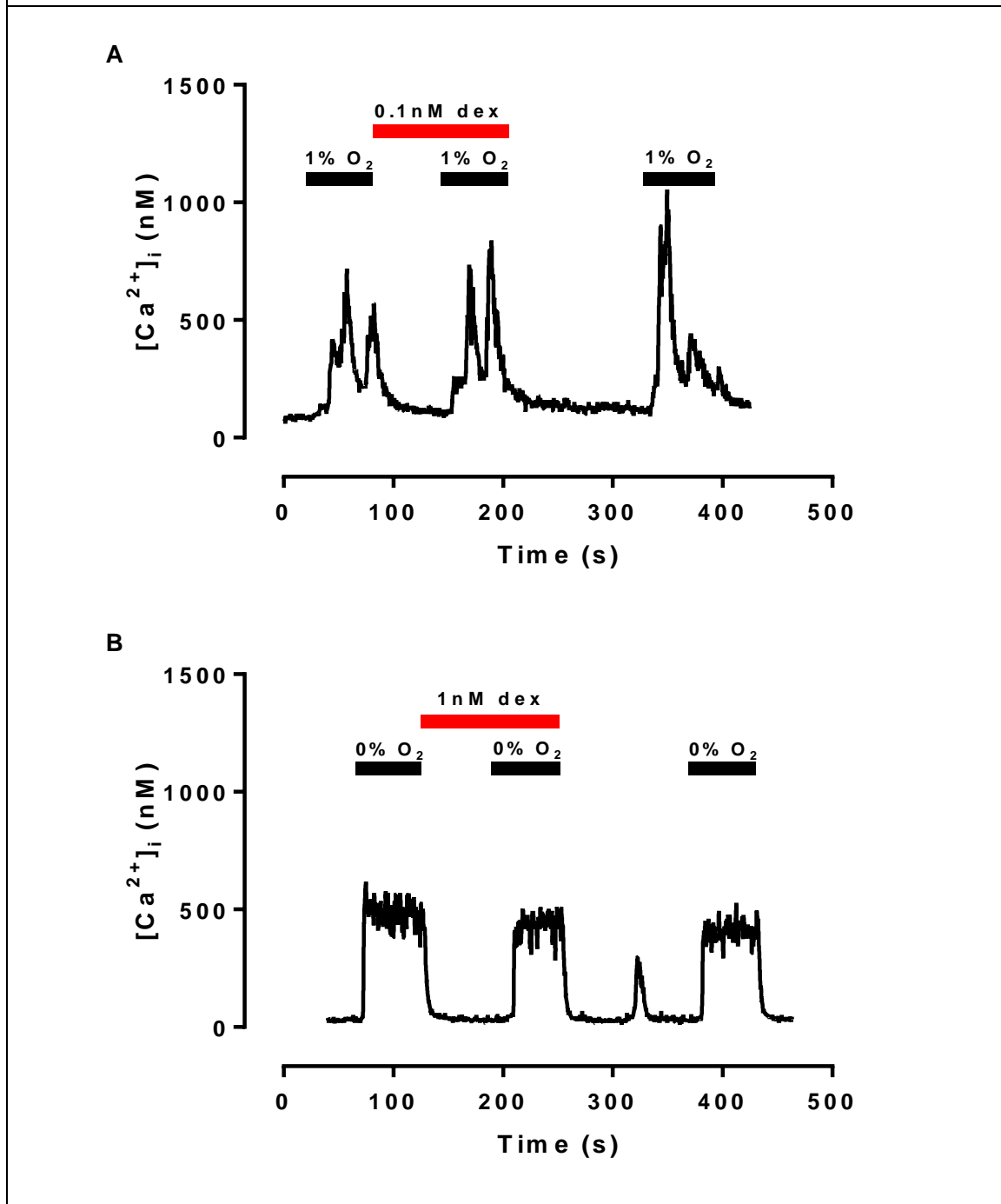
Hypoxia increased  $[Ca^{2+}]_i$  and as expected in the absence of dexmedetomidine, severe hypoxia elicited a greater increase in mean (SEM)  $[Ca^{2+}]_i$  than mild hypoxia in rat glomus cells ( $189 \pm 41$  nM;  $n=21$ , versus  $95 \pm 41$  nM;  $n=9$   $p=0.009$ ). However, the magnitude of increase in  $[Ca^{2+}]_i$  evoked by mild and severe hypoxia was not influenced by dexmedetomidine (ANOVA,  $p=0.512$ ) and there was no interaction between level of hypoxia and drug ( $p=0.544$ ).

For resting  $[Ca^{2+}]_i$  ANOVA suggested a significant influence of the interactive term of 'drug' and 'dose' ( $p = 0.026$ ) and post hoc testing confirmed the marginally, but significantly, decreased values after exposure to high dose

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dexmedetomidine ( $72 \pm 14$  nM control;  $n=30$ , versus  $55 \pm 12$  nM drug;  $n=21$ ,  $p=0.018$ ), but not low dose ( $72 \pm 14$  nM control;  $n=30$ , versus  $98 \pm 33$  nM drug;  $n=9$ ,  $p=0.314$ ).

**Figure 5.2** Example traces of intracellular  $[Ca^{2+}]_i$  glomus cell responses to ~60 sec exposures to hypoxia; **A.** mild hypoxia **B.** severe hypoxia. The more severe hypoxia elicits a greater response and there is no apparent influence of dexmedetomidine in either hypoxic exposure.



### **Acute hypoxic ventilatory response in mice with dexmedetomidine administration**

Figure 5.3 shows a typical plethysmographic output of mice breathing quietly in euoxia, and the effect of hypoxia, with and without dexmedetomidine. Minute ventilation rose abruptly, as expected, with hypoxia and even during this short exposure, some 'hypoxic ventilatory decline' is evident with sustained hypoxia (a phenomenon modelled elsewhere; (Bascom et al., 1990)).

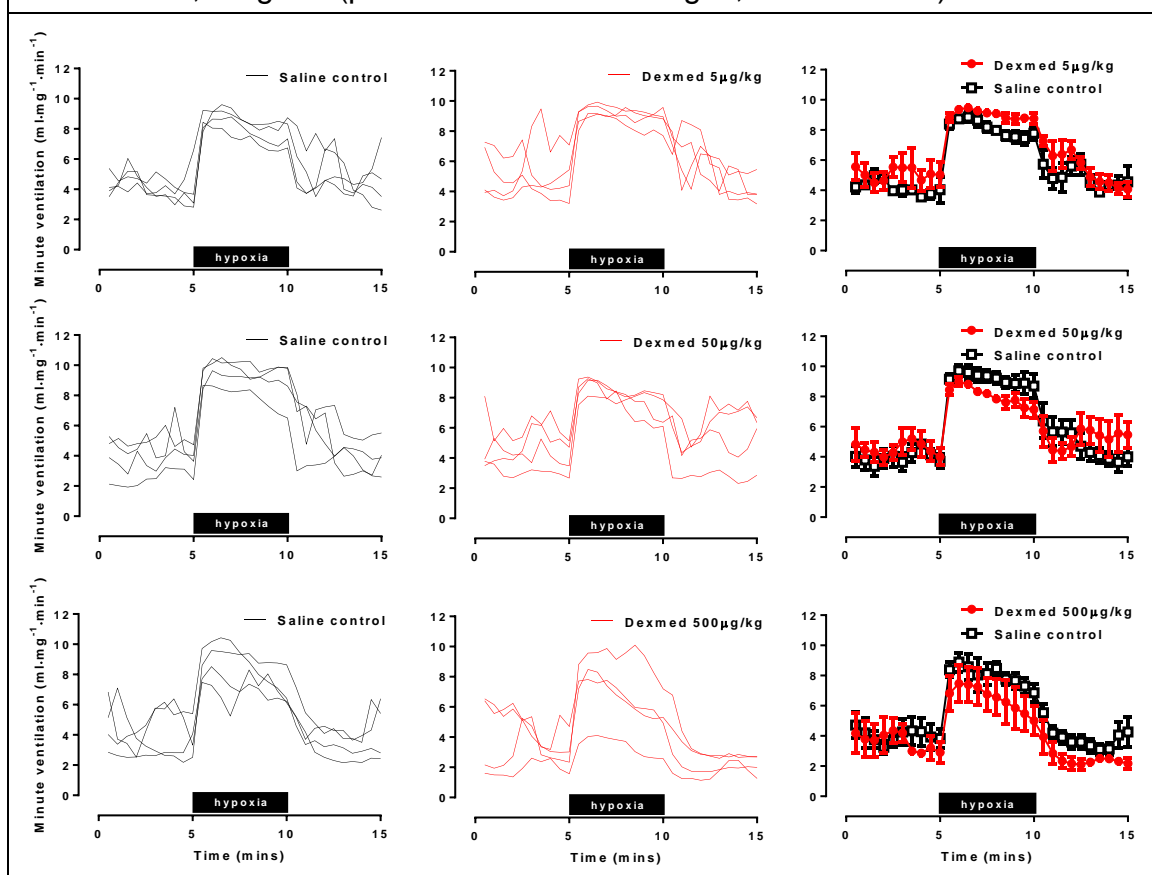
During this short exposure, some 'hypoxic ventilatory decline' (HVD) is evident with sustained hypoxia (a phenomenon recognised in rodents (Bissonnette and Knopp, 2001; Maxova and Vizek, 2001) and modelled elsewhere (Bascom et al., 1990)). This HVD gives the appearance of a depressive influence of dexmedetomidine on ventilation, so we calculated its magnitude (Table 5.1). Although there is an increase in magnitude of HVD with dose of dexmedetomidine, this did not reach statistical significance ( $p=0.127$ ). There is appropriate return of ventilation to pre-hypoxic levels on return to euoxia. Also evident is the variability in control (no dexmedetomidine) responses between the groups of mice for each experiment, underlining the importance of using each group of mice as its own control for the drug experiments. Overall, any effect of dexmedetomidine seems at best modest. Only at the highest dose is there a suggestion of a reduced response during hypoxia, but this must be offset against what appears to be a small reduction in baseline ventilation.

Figure 5.4 shows the quantitative analysis and averaged results for all mice across the three protocols for both pre-hypoxic euoxic ventilation and AHVR. Analysis of variance confirmed that dexmedetomidine had no significant effect

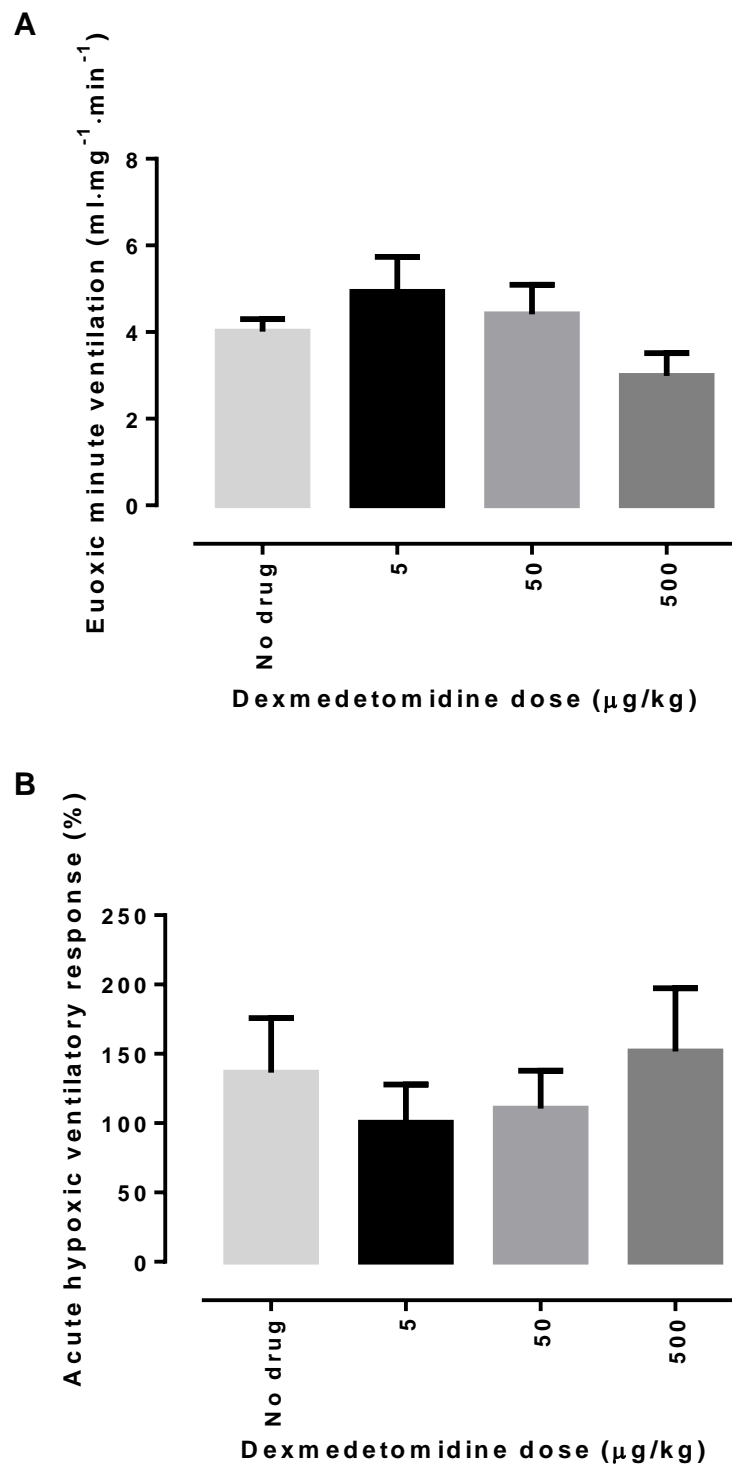
Chapter 5 - The influence of dexmedetomidine on glomus cell and rodent response to hypoxia

on resting minute ventilation in mice while breathing air at any dose delivered compared with control, ( $p=0.291$ ). Although in Figure 5.3 it might appear that low dose dexmedetomidine augments AHVR while higher doses depress this, there were no statistically significant dose dependant effects on normalised ( $p=0.559$ ) or absolute hypoxic ventilatory response ( $p=0.997$ ).

**Figure 5.3** Influence of dexmedetomidine on hypoxic responses in mice. Each row shows the results for one drug concentration: top to bottom, from low dose, middle dose and high dose dexmedetomidine. The first column is the control data for that dose, the middle column the with-drug data. In these first two columns, each trace shows the data for a single mouse (individual data points omitted for clarity). The last column are the averages for the drug dose, control values black, drug red (points are 30-sec averages, mean  $\pm$  SEM).



**Figure 5.4** Quantitative analysis of dexmedetomidine effect on **A.** Euoxic breathing in mice, though there appears to be a dose dependant decrease in minute ventilation, this was not statistically significant. **B.** The AHVR (%) of mice was not effected over a large (5-500  $\mu\text{g}/\text{kg}$ ) concentration range. All data are mean  $\pm$  SEM, 4 animals per group.



**Table 5.1** Quantification of hypoxic ventilatory decline by dexmedetomidine dose.

Condition	HVD (% $\pm$ SEM)	n
No drug (control)	22 $\pm$ 6	12
Dexmed 5 $\mu$ g/kg	13 $\pm$ 7	4
Dexmed 50 $\mu$ g/kg	34 $\pm$ 17	4
Dexmed 500 $\mu$ g/kg	51 $\pm$ 25	4

### **Discussion**

The main results of this study are that: (1) dexmedetomidine has no effect (i.e., neither stimulation nor depression) on the glomus cell response to mild or severe hypoxia; and (2) in intact and unrestrained mice, bolus dosing of dexmedetomidine had no significant effect on euoxic minute ventilation in air, or on the response to hypoxia.

Thus, while I conclude that dexmedetomidine is unlikely to be a depressive drug to the ventilatory chemoreflex (as was suggested by Nishida et al. (2002) and Fernandes et al. (2006)), I would not go as far as to suggest that it actually stimulates chemoreflex function (as was suggested by Nguyen et al. (1992) and Nakatani et al. (2012)). This 'neutral' effect nevertheless implies a potentially beneficial therapeutic profile for clinical practice, since during sedation or anaesthesia it is highly desirable to maintain chemoreflex function as a protective response.

Even in theory it is not fully established how  $\alpha$ 2-adrenoceptors (the main putative target for dexmedetomidine) could influence the hypoxic chemoreflex loop. The same study that identified  $\alpha$ 2-adrenoceptors existing on glomus cells in rabbits (Kou et al., 1991), showed that the guanabenz (like dexmedetomidine,

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an  $\alpha_2$ -adrenoceptor agonist), reduced carotid body hypoxic chemosensory discharge. However, this study was conducted in an intact preparation without control of local blood flow or blood pressure, and it is possible that these might have influenced the result (noting that Nakatani et al. (2012) reported the opposite effect for dexmedetomidine).

It was a strength of this study that the findings were replicated across two species (rats and mice) and across several doses of drug and levels of hypoxia. Any whole animal work makes it difficult to establish whether carotid body, central neural, or haemodynamic effects predominate in the effect (or non-effect) of a drug, but studying isolated glomus cells, as I did, enables us to draw clearer conclusions about the direct effects of drug on the primary chemoreceptor. I did not, however, use carotid bodies from mice. Isolation of glomus cells from mice is technically extremely challenging and although our group has reported on the results of such investigations, this has been after the culmination of several years of work (Turner and Buckler, 2013). Had the rat glomus cell experiments yielded a positive result, then that is something that might be considered worthwhile. I might have also studied rats in the plethysmography experiment but a lack of finding of effect for the drug in either isolated glomus cells or in intact mice makes it very unlikely that intact rats alone would produce a different result. The results here, therefore, should be seen as applying to rodents in general.

In the plethysmography experiments, I was careful to employ real time capnography to ensure that hypoxic input was stable over time and equivalent across the protocols. Although I do not report on hypercapnic responses, we did

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employ the technique of adding 3% CO<sub>2</sub> to the inspire to ensure constancy of CO<sub>2</sub> (isocapnia) during the hypoxic exposures (as otherwise there would have been hypocapnia, which would have acted as a brake to ventilatory response, and therefore a confounder).

**Limitations**

This methodology was predominantly directed to assessing the peripheral chemoreceptor contribution to acute, rather than sustained respiratory responses. Were dexmedetomidine administered in human studies a depressive effect is conceivable through a direct central action of the drug. This is what Danielson et al. (2014) may have observed in a preliminary study showing depressed hypoxic ventilatory response in humans with dexmedetomidine. Our observation of enhanced HVD with increasing doses of dexmedetomidine is perhaps one sign that such central influences may be at play, even in our model. Whereas in humans, HVD may originate in the peripheral chemoreceptor (Robbins, 1995), in animals there is compelling evidence that it may be of central origin (Ward et al., 1990). Normally HVD is studied over hypoxic exposures of 20-30 min, whereas our methods used hypoxic exposure time of only 5 min: if dexmedetomidine has a pronounced effect in increasing HVD our methodology would not have detected this. Therefore, future human studies should explore the effect of drug in both acute and sustained hypoxia.

Belleville et al. (1992) studied hypercapnic (but not hypoxic) chemoreflexes, reporting a depressive effect on CO<sub>2</sub>-induced stimulation of breathing (an effect in contrast to what we might predict from these results).

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The finding that dexmedetomidine is likely to be neutral in its effects, or even stimulates the chemoreflexes, raises the intriguing possibility that, when combined with other anaesthetic drugs (e.g. volatiles or intravenous as an 'anaesthetic sparing' drug) it might reverse or prevent the depressive effect of the latter on chemoreflex function.

In summary I have demonstrated that:

- Dexmedetomidine does not influence the glomus cell  $[Ca^{2+}]_i$  response to severe or mild hypoxia.
- In sub-sedative doses, dexmedetomidine has a neutral effect on unrestrained murine AHVR.

**Chapter 6:**

**STIMULATION OF THE CAROTID BODY GLOMUS CELL  
RESPONSE TO HYPOXIA BY TASK CHANNEL BLOCKERS**

## **Overview**

The physiological response to systemic hypoxia is to increase the minute ventilation to restore normal arterial oxygen tension and maintain oxygen delivery to tissues (Weir et al., 2005). As arterial blood oxygen tension drops, the hypoxia triggers a sequence of events that cause  $K_{2P}$  channels expressed in carotid body glomus cells to close (Buckler and Vaughan-Jones, 1994a; Buckler, 1997; Turner and Buckler, 2013; Buckler, 2015), decreasing the background potassium current and depolarising the membrane. With this change in membrane potential, voltage-gated calcium channels ( $Ca^{2+}_v$ ) are activated and calcium enters the cell (Buckler and Vaughan-Jones, 1994a), signalling neurotransmitter release (Kåhlin et al., 2014). One or more neurotransmitters (Fidone et al., 1982; Fitzgerald et al., 1999; Vandier et al., 1999; Vicario et al., 2000; Shirahata et al., 2007) then bind to the carotid sinus nerve terminal and evoke neuro-afferent discharge to the respiratory centres of the brain. The physiological result of this reflex loop is the increase in minute ventilation called the acute hypoxic ventilatory response (AHVR).

There are many situations when this reflex loop is interrupted, one being the use of volatile general anaesthetics. Volatile anaesthetics depress the peripheral limb of the AHVR. By de-afferenting the sensory input, a significant decrease in both the acute hypercapnic response (AHCR) (Dahan et al., 1994; van den Elsen et al., 1998; Pandit et al., 1999b; Pandit, 2005) and AHVR (Knill and Gelb, 1978; Nagyova et al., 1994; Nagyova et al., 1995; Pandit et al., 1999a; Pandit, 2002) is observed, even at sub-hypnotic concentrations (Dahan et al., 1994; Nagyova et al., 1994; Nagyova et al., 1995; van den Elsen et al.,

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1995; Pandit et al., 1999a). Respiratory depression occurs commonly in the post-operative setting (Craig, 1981; Rodgers et al., 2000; Myles et al., 2007; Sun et al., 2015), as an enduring side-effect of general anaesthesia (Wong et al., 1995) and also the use of opioid analgesics (Lee et al., 2015).

The mechanism by which volatile anaesthetics obtund the AHVR is contentious, as it could occur at any level of the reflex loop. A central site of action is plausible, as audiovisual stimulation can influence the anaesthetic depression of AHVR, though this is not consistent across all agents (Pandit, 2002), so cannot be the only responsible mechanism. Consistency arises at the level of the peripheral chemoreceptor, where halothane, sevoflurane (Pandit and Buckler, 2009), and isoflurane (Pandit and O’Gallagher, 2008) all inhibit hypoxia evoked calcium influx into glomus cells. This is likely to be explained by the action of volatile anaesthetics on TASK channels, at least for halothane (Buckler et al., 2000) and isoflurane (Pandit et al., 2010b). Unpublished data from this laboratory, suggests the same will be demonstrated for sevoflurane (Huskens, Thesis 2015, unpublished).

As such, one strategy to combat anaesthetic depression of AHVR is to stimulate the carotid body using other drugs. This would have the potential to reduce the harm caused by hypoxaemia post operatively. Also, since respiratory depressants act synergistically with anaesthetics, this would reduce the concerns about using analgesics such as opioids, which also depress ventilation, albeit centrally.

Doxapram is the only ventilatory stimulant in current clinical practice. It is of utility in increasing breathing, but carries a significant side-effect profile,

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including hypertension, tachycardia, and raised intracranial pressure (Yost, 2006). This compound has been shown to have a direct effect on glomus cells (Peers, 1991), causally explaining its ventilatory stimulant effect. It is however non-specific, acting on TASK-1 and TASK-3 (Cotten et al., 2006), as well as the  $\text{Ca}^{2+}$ -activated potassium channel BKCa (Peers, 1991). It is this lack of specificity that characterises drugs with off target side-effects. Almitrine, until recently a widely used respiratory stimulant, was withdrawn for just this reason as while effective at stimulating the peripheral chemoreceptors and breathing (Laubie and Schmitt, 1980; Laubie et al., 1983, 1984), it caused unacceptable neuropathy and weight loss (European Medicines Agency, 2013).

Two recently described compounds exhibit much greater specificity for TASK channels. PK-THPP has been described as a potential treatment for neurological diseases and displays specificity as an antagonist of TASK 3 (Coburn et al., 2012). A1899 conversely has been shown to be a specific antagonist for TASK 1 (Streit et al., 2011) when expressed in oocytes. It has been demonstrated that specific antagonism of the TASK 1 and TASK 3 (by A1899 and PK-THPP respectively) causes stimulation of ventilation in isoflurane anaesthetised rats (Cotten, 2013), far more potently than doxapram. As  $\text{K}_{2\text{P}}$  channels are expressed widely in neuronal tissue, it is unclear whether this stimulation of breathing was due to a peripheral, central or efferent drug effect.

A plausible explanation of the stimulating effect of  $\text{K}_{2\text{P}}$  channel antagonists is that they stimulate glomus cells directly, by causing membrane depolarisation. If this is the case, because they are acting in a similar manner to hypoxia, it would be possible to decrease the threshold for excitation of glomus cells by hypoxia.

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The mechanisms by which volatile anaesthetics act on  $K_{2P}$  channels are not understood. Mutation analysis suggests that certain amino-acid residues (Conway and Cotten, 2012) and C-terminus domains (Patel et al., 1999) are critical regulatory sites on TASK channels for anaesthetic binding and action. If these mechanistic insights are correct, TASK 1 and TASK 3 would still be effected by the pore blockers A1899 and PK-THPP respectively, despite volatile anaesthesia.

TASK 1 has been reported to be relatively insensitive to isoflurane (Putzke et al., 2007). However, it has been demonstrated that the native TASK current in rat glomus cells is relatively sensitive to isoflurane (Pandit et al., 2010b). This may be due to the effect on TASK 3, or the heteromultimer of TASK 1 and TASK 3. It has been suggested that TASK 1/3 heteromultimers have a reduced affinity for A1899 (Rinné et al., 2015). With this information, it may be reasonably deduced that by antagonising TASK 1, glomus cells could be pharmacologically excited by A1899 in the presence of isoflurane.

I therefore hypothesised that:

- (1) Both A1899 and PK-THPP would stimulate glomus cells.
- (2) This stimulation would persist in the presence of volatile anaesthetics.
- (3) At appropriate concentrations, both A1899 and PK-THPP would augment the glomus cell response to weak hypoxia.
- (4) Doxapram would also stimulate glomus cells, but at higher concentrations.

## **Methods**

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986.

### **Cell isolation**

Carotid bifurcations were dissected in situ from neonatal Sprague-Dawley rats (P11-14, Harlan, Blackthorn, Oxfordshire, UK) under terminal isoflurane anaesthesia and placed in ice-cold saline. As described in Chapter 2 – General Methods, primary cell cultures were plated onto coverslips and incubated for two hours prior to loading with indo-1, AM dye for 60 minutes. Primary cell cultures were pooled and also prepared for use in unrelated experiments concurrently.

### **Calcium imaging**

An inverted microscope (Nikon Diaphot, Kingston, Surrey, UK) with 40x oil-immersion objective was used to identify glomus cells, which were excited at a wavelength of 340 nm by filtered light from an arc lamp. In epifluorescence mode, emission light was split and measured at dual emission peaks of 405 nm and 495 nm by two trialkali photomultiplier tubes (Thorn EMI, London, England, UK). Intracellular calcium concentration was two-point calibrated in situ as described in Chapter 2 – General Methods.

### **Hypoxia experiments.**

Each cell was sequentially superfused with warmed Tyrode's solution (in mM: 117 NaCl, 4.5 KCl, 1 MgCl<sub>2</sub>, 23 NaHCO<sub>3</sub>, 11 glucose). equilibrated with 5% CO<sub>2</sub> in balanced air (euoxia) and 5% CO<sub>2</sub> in balanced N<sub>2</sub> (severe hypoxia) to identify glomus cells, 5% CO<sub>2</sub>, 1% O<sub>2</sub> in balanced N<sub>2</sub> (moderate hypoxia), 5%

## *Chapter 6 - Stimulation of the carotid body glomus cell response to hypoxia by TASK channel blockers*

CO<sub>2</sub>, 2% O<sub>2</sub> in balanced N<sub>2</sub> (mild hypoxia). Severe hypoxia (0% O<sub>2</sub>) measured using a fluorescence quenching optode, was <3 mmHg O<sub>2</sub>, designed to elicit robust activation of cells. Moderate hypoxia (1% O<sub>2</sub>, 15 mmHg O<sub>2</sub>) was used in the case where it was expected that an augmentation or depression of cellular response to hypoxia may occur, and mild hypoxia (2% O<sub>2</sub>, 25 mmHg O<sub>2</sub>) where the postulate was augmentation alone. Where isoflurane was used, a calibrated agent-specific vaporizer (Penlon, Abingdon, UK) was used to bubble 1.5% (as measured by headspace infra-red analysis, Capnomac Ultima, Datex, Helsinki, Finland) continuously into the salt solution.

### **Drug experiments**

After identification, cells were superfused with the salt solution described in the presence or absence of drug, and always as their own control. Concentrations of A1899 and PK-THPP between 12 and 1200 nM were studied, in the presence or absence of isoflurane 1.5%.

Drugs were applied over 60 seconds, with a similar wash period following exposure. A1899 and PK-THPP were supplied by Aberjona Laboratories (Boston, MA, USA), doxapram by Abcam (Cambridge, Cambridgeshire, UK) and isoflurane by Meriel (Harlow, Essex, UK)

### **Statistical analysis**

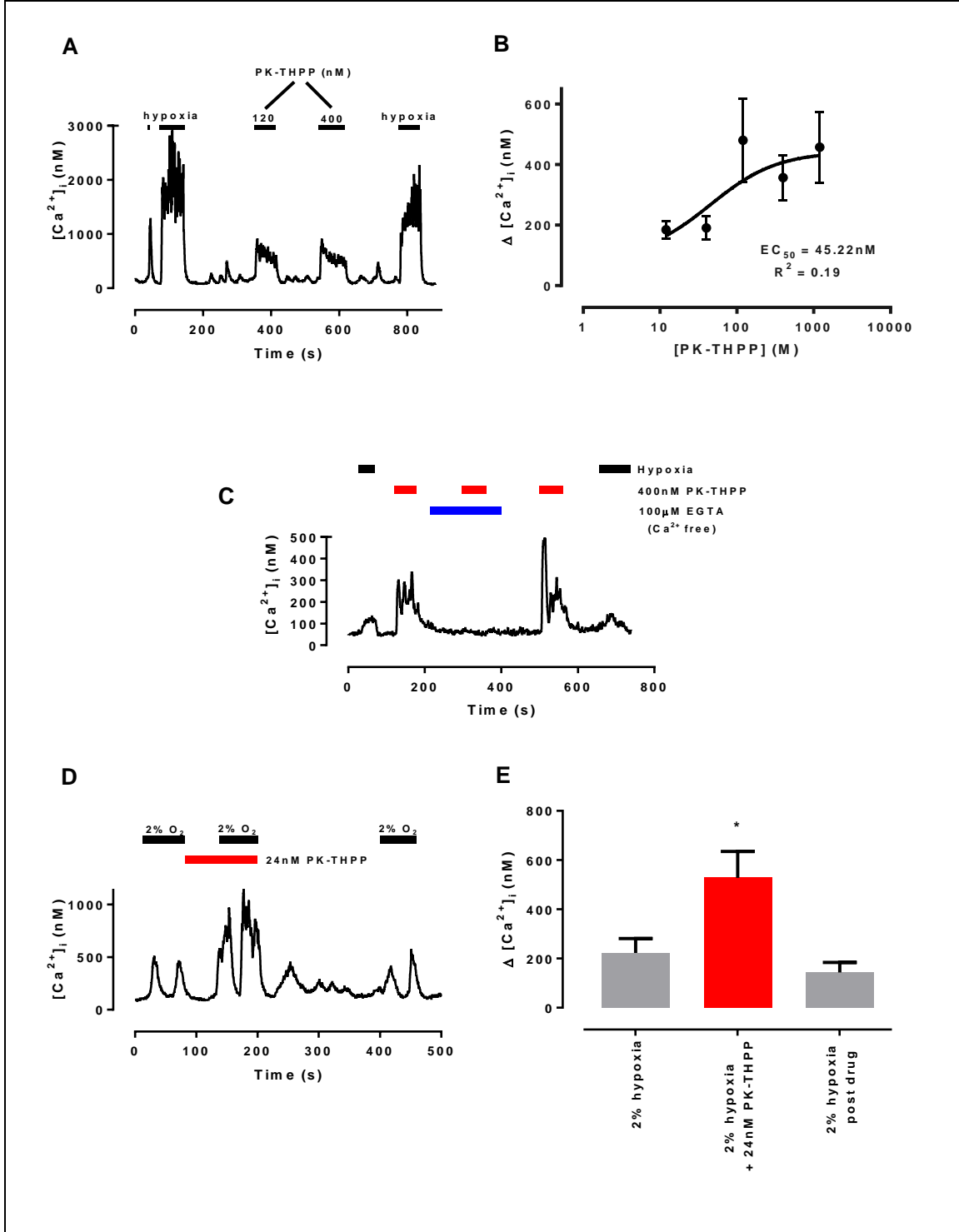
Where paired data were compared, a paired Student's t-test was used. Where more than two groups were compared, ANOVA was used. Correlation of concentration and response was analysed using Spearman's rank correlation coefficient.

## **Results**

### **Glomus cell $[Ca^{2+}]_i$ response to PK-THPP application**

Application of PK-THPP evoked a rapid rise in  $[Ca^{2+}]_i$  in a manner similar to severe hypoxia (Figure 6.1A). PK-THPP evoked an increase in glomus cell  $[Ca^{2+}]_i$  across a range of concentrations (12-1200 nM), from a mean of  $126 \pm 11$  to  $324 \pm 40$  nM ( $n=35$ ,  $p < 0.001$ ). Quantitatively increasing concentration of PK-THPP correlated with  $[Ca^{2+}]_i$  (Spearman's Rho = 0.359,  $p=0.034$ ), Figure 6.1B. Drug evoked changes in  $[Ca^{2+}]_i$  were abolished with  $Ca^{2+}$  free EGTA solution (Figure 6.1C). PK-THPP 24 nM augmented the  $[Ca^{2+}]_i$  increase evoked by weak hypoxia  $223 \pm 58$  vs  $528 \pm 108$  nM ( $p=0.008$ ), Figure 6.2D-E.

**Figure 6.1** The effect of PK-THPP on glomus cell  $[Ca^{2+}]_i$ . **A.** Representative trace of application of various drug concentrations. **B.** Quantitative responses over the range of concentrations used. Each point represents mean  $\pm$  SEM and the curve is fit by non-linear least squares regression to a variable slope model. **C.** Effect of  $Ca^{2+}$  free Tyrode on responses evoked by PK-THPP. **D.** PK-THPP effect on glomus cell hypoxic response. Values are mean  $\pm$  SEM. **E.** Quantitative effect of PK-THPP on the glomus cell response to mild hypoxia (with vs without drug,  $p = 0.008$ ).



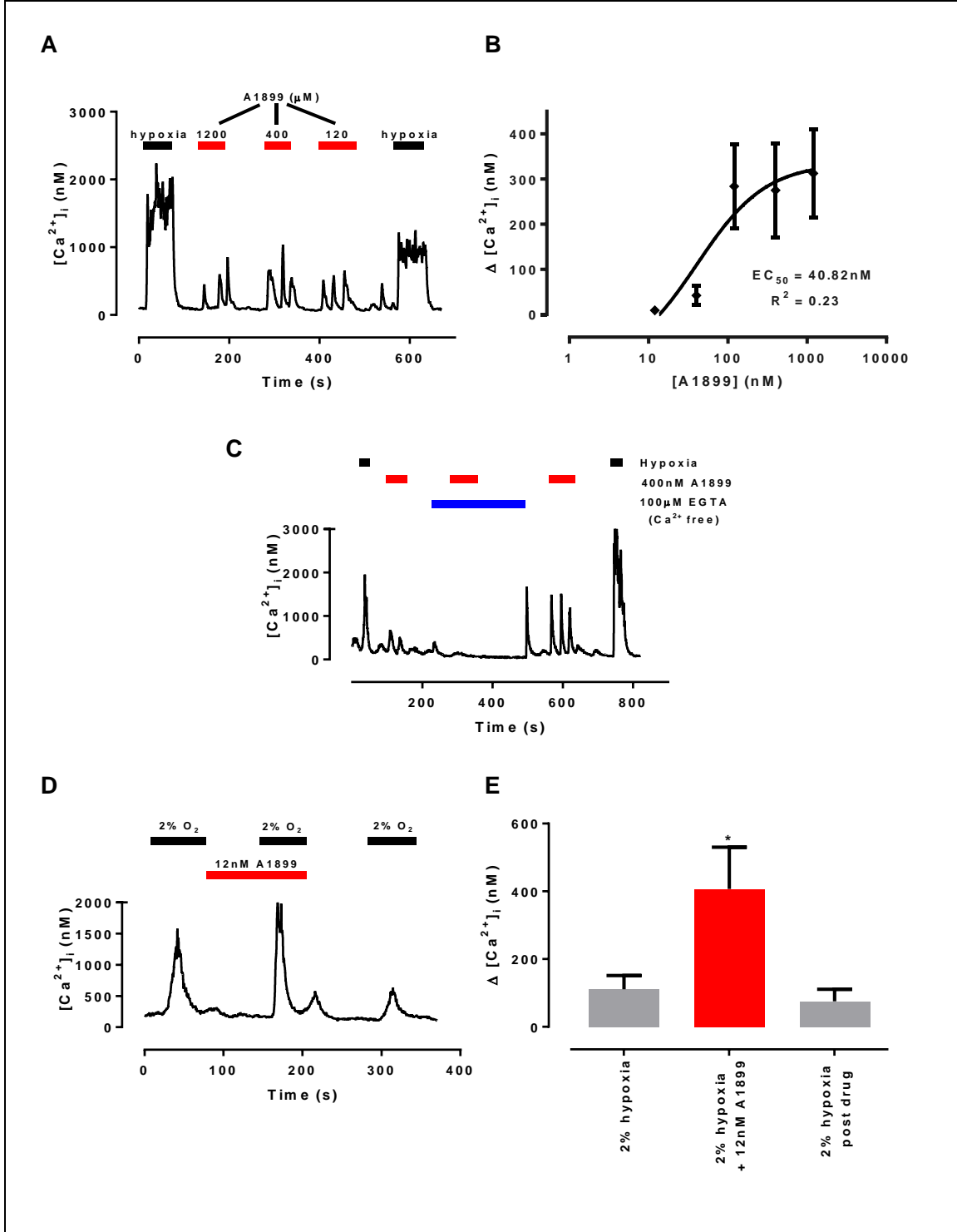
### **Glomus cell $[Ca^{2+}]_i$ response to A1899 application**

Across the same concentration range (12-1200 nM), A1899 caused an increase in  $[Ca^{2+}]_i$  from a mean of  $112 \pm 9$  nM to  $315 \pm 44$  nM ( $n=36$ ,  $p < 0.001$ ). Whereas hypoxia elicits a sustained increase in  $[Ca^{2+}]_i$  over the period of exposure, A1899 often caused “spiking” (Figure 6.2A and Figure 6.4A) of the  $[Ca^{2+}]_i$ , reminiscent of the pattern seen when cells are exposed to  $CO_2$  or barium (Pandit et al., 2010a). Concentration of A1899 correlated with an increase in  $[Ca^{2+}]_i$  (Spearman’s Rho = 0.707,  $p < 0.001$ , Figure 6.2C). The increase in  $[Ca^{2+}]_i$  evoked by A1899 was abolished when cells were superfused in a  $Ca^{2+}$  free EGTA solution (Figure 6.2C). A1899 12 nM augmented the  $[Ca^{2+}]_i$  increase evoked by weak hypoxia  $111 \pm 41$  vs  $407 \pm 124$  nM ( $p = 0.035$ ), Figure 6.2D-E.

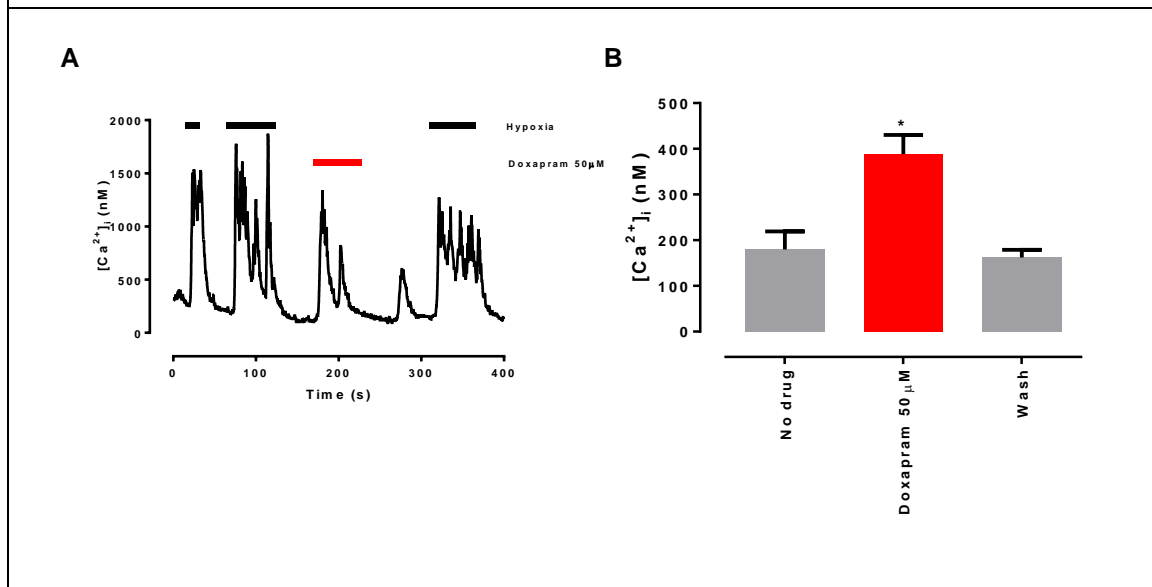
### **Glomus cell $[Ca^{2+}]_i$ response to doxapram application**

It was confirmed that as expected, doxapram 50  $\mu$ M caused a rapid and reversible increase in  $[Ca^{2+}]_i$  in glomus cells (Figure 6.3A). This drug evoked change was significant, on average  $[Ca^{2+}]_i$  increased from  $180 \pm 40$  to  $388 \pm 42$  nM ( $n=6$ ,  $p < 0.001$ , Figure 6.3B)

**Figure 6.2** The effect of A1899 on glomus cell  $[Ca^{2+}]_i$ . **A.** Representative trace of application of various drug concentrations. **B.** Quantitative responses over the range of concentrations used. Each point represents mean  $\pm$  SEM and the curve is fit by non-linear least squares regression to a variable slope model. **C.** Effect of  $Ca^{2+}$  free Tyrode on responses evoked by A1899. **D.** A1899 effect on glomus cell hypoxic response. Values are mean  $\pm$  SEM. **E.** Quantitative effect of A1899 on the glomus cell response to mild hypoxia (with vs without drug,  $p=0.03$ ).



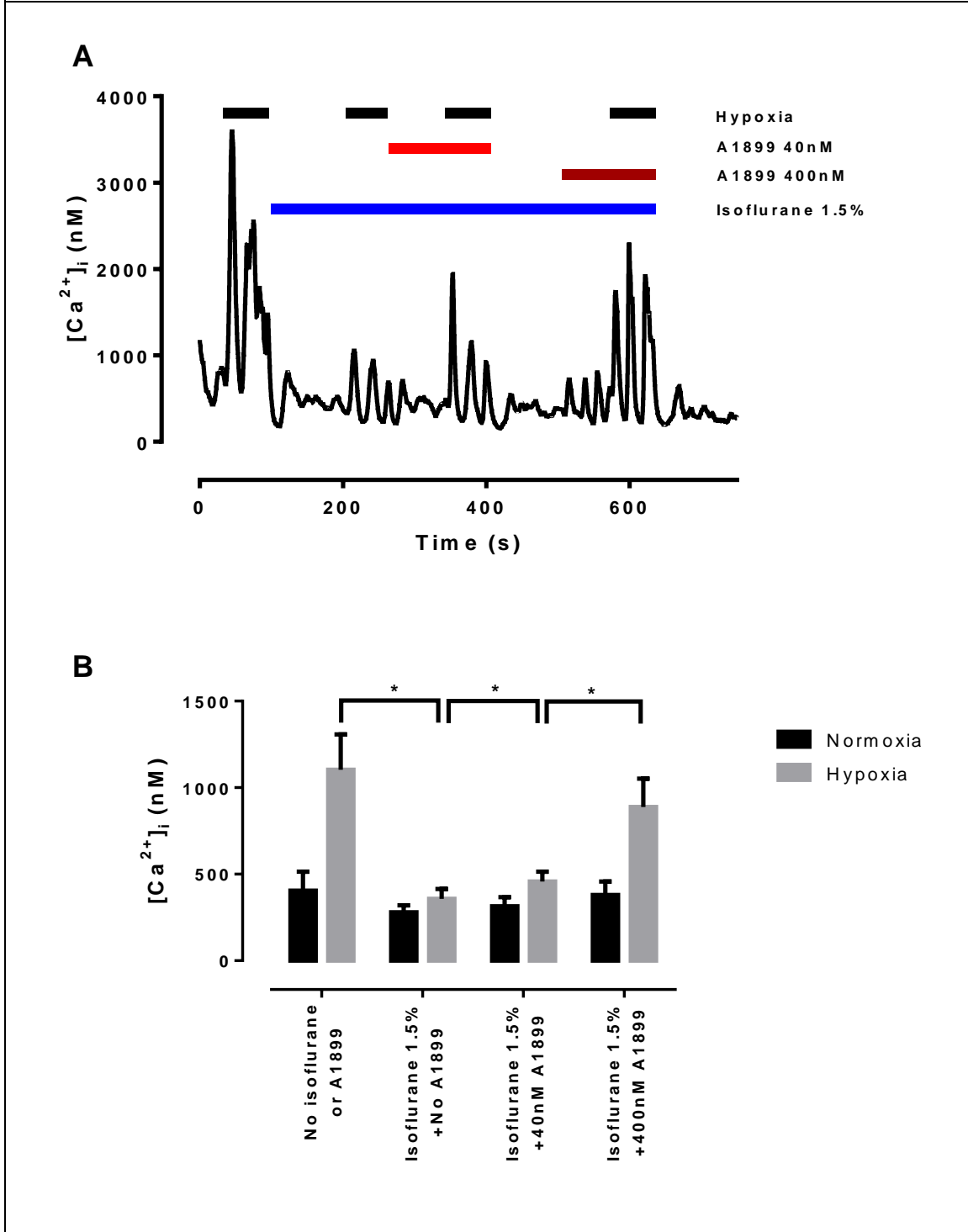
**Figure 6.3** Effect of doxapram on glomus cell  $[Ca^{2+}]_i$  **A.** Representative trace of the effect doxapram on glomus cell  $[Ca^{2+}]_i$  **B.** Quantitative effect of doxapram on glomus cell  $[Ca^{2+}]_i$  drug vs no drug,  $p < 0.001$ . Mean  $\pm$  SEM.



#### Glomus cell response to A1899 in the presence of isoflurane 1.5%

Isoflurane 1.5% had a depressive effect on glomus cell hypoxia induced  $[Ca^{2+}]_i$ , from a mean increase of  $698 \pm 196$  to  $77 \pm 35$  nM ( $n=5$ ,  $p < 0.001$ ) when exposed to 1% oxygen (Figure 6.4A). A1899 also had a significant effect on  $[Ca^{2+}]_i$ , increasing  $[Ca^{2+}]_i$  from  $281 \pm 39$  to  $348 \pm 45$  nM in normoxia and  $358 \pm 57$  to  $673 \pm 109$  nM in 1% hypoxia (ANOVA,  $p=0.001$ ), in a dose dependant manner (ANOVA,  $p=0.042$ ). Combining the interactive terms for isoflurane, A1899 and 1% hypoxia was also significant ( $p=0.002$ ), suggesting that A1899 antagonised the observed depression of hypoxic response by isoflurane (Figure 6.4B).

**Figure 6.4** A1899 has an enduring effect on hypoxia evoked increase in  $[Ca^{2+}]_i$  in the presence of isoflurane 1.5%. **A.** Representative trace of the depressive effect of isoflurane on glomus cell hypoxia evoked  $[Ca^{2+}]_i$  and partial restoration with A1899. **B.** Quantitative relationship between hypoxia induced increase in  $[Ca^{2+}]_i$ , the depression with isoflurane 1.5% and partial restoration with A1899.



## **Discussion**

I have demonstrated that two TASK channel antagonists A1899 (TASK 1) and PK-THPP (TASK 3) can directly stimulate carotid body glomus cells. Secondly, these compounds are effective in exciting glomus cells even in the presence of commonly used volatile anaesthetics (prototypical TASK agonists and depressants of chemosensation). Perhaps of greater significance is the discovery that when applied in concentrations insufficient to cause significant calcium entry into glomus cells, both drugs augmented the cellular response to hypoxia. This turned a weak chemostimulus into one of much greater magnitude, turning up the “gain” of the peripheral chemosensor.

The EC<sub>50</sub> values for increase in [Ca<sup>2+</sup>]<sub>i</sub> were 41 nM (A1899) and 45 nM (PK-THPP). Previously reported *inhibitory* drug effects of A1899 for TASK 1 channel activity (which leads to glomus cell *excitation*) are 7-35 nM depending on the expression system used (Streit et al., 2011). The higher value agrees favourably with my observation in native cells. For PK-THPP the values for TASK 3 are 35-42 nM (Coburn et al., 2012; Cotten, 2013), again showing good agreement with this study. A significant strength of the data presented here is the use of native cells. The expression pattern of TASK 1 and TASK 3 is complex, with both homomeric and heteromeric channels likely to form part of the oxygen sensitive potassium conductance (Turner and Buckler, 2013). It is generally agreed that the heteromeric TASK 1/3 channel is the predominant functional form of the TASK conductance (Kim et al., 2009). Recent studies have cautioned against drawing conclusions from the pharmacological probing of TASK channels in expression systems (Rinné et al., 2015). In the context of

human atria, the use of native cardiomyocytes revealed evidence of TASK 3 preferentially assembling as a heteromultimer with TASK 1 (Rinné et al., 2015), to the point where TASK 3 homomeric channel activity was not detectable. Rinné et al. (2015) also commented that the response of concatameric channels to specific TASK antagonists (including A1899) is influenced by how they have been linked (T1-T3 or T3-T1). That is to say, by artificially constraining a how the subunits form a channel, the sensitivity to A1899 can be altered. Not only this, but one of the few  $K_{2P}$  channels that have a solved crystal structures to date (TRAAK) has been shown to exhibit domain swapping (Brohawn et al., 2013), where during the formation of a functional channel, parts of one protein subunit are replaced with those of another subunit. This process may be key to function (Renigunta et al., 2015). Replicating these behaviours in expressed channel systems has not been demonstrated.

To this end, it is important that we have established the functional utility of these TASK antagonists in native cells, expressing channels in their normal milieu.

In showing that A1899 is effective in stimulating glomus cells in the presence of a robust concentration of isoflurane, I have extended the results of other groups (e.g. Cotten, 2013) by bridging the gap between the study of expressed TASK channels, and in-vivo observations in anaesthetised animals. Isoflurane is a commonly used anaesthetic, and a desirable feature of any novel ventilatory stimulant is that it is efficacious in the peri-operative period, when the volatile agents linger in significant concentrations (Lockwood, 2010).

We also advance the potential clinical utility of TASK channel antagonists A1899 and PK-THPP, by showing that they can *augment* chemosensitivity. This

is mechanistically plausible through a partial blocking (sub-IC50) action on the main determinant of membrane potential (TASK), rendering cells more excitable. At a particular concentration (24 nM or 12 nM for PK-THPP and A1899 respectively) the excitatory effect *only* occurs when hypoxia is co-applied. In a clinical scenario, it could therefore “heighten the senses” of the chemosensory apparatus. The “pharmacological ventilator” doxapram, currently the only available respiratory stimulant has to be used in high concentration infusions to achieve its function. I report here that to achieve a similar increase in  $[Ca^{2+}]_i$  as PK-THPP or A1899 (compare Figure 6.1 with Figure 6.2 with Figure 6.3), a concentration of 50  $\mu$ M doxapram was required. The nanomolar concentrations we applied to achieve all of the observed effects with selective TASK antagonists would potentially reduce the deleterious side effects that are observed with doxapram.

In summary I have demonstrated that :

- A1899 and PK-THPP excite glomus cells in a concentration dependant manner
- Doxapram evokes a significant increase in  $[Ca^{2+}]_i$  in glomus cells, though at a much higher concentration than both PK-THPP and A1899.
- A1899 can excite these cells in the presence of isoflurane, and both PK-THPP and A1899 can augment the cellular response to a mild hypoxic stimulus.

**Chapter 7:**

**DISCUSSION AND CONCLUSIONS**

**General conclusions**

This thesis provides further evidence of the role of TASK channels not only in generating the AHVR, but also implicating them in the anaesthetic depression of this mechanism. Chapter 3 has shown that both TASK 1 and TASK 3 are required for normal chemosensation in mice, supporting the heteromultimeric form of the channel as the predominant functional channel. Individually knocking out each channel results in differential depression of AHVR by isoflurane and halothane, suggesting not only that the agents may act dissimilarly on the isoforms of the channel, but also that modulating channel function could be a pharmacological target for reversing these deleterious effects.

Chapter 4 of this thesis has also established that propofol depresses the carotid body *glomus cell* response to hypoxia and hypercapnia. Previous work has postulated a post-synaptic anatomical location for this drug action. This is the first report of the action of propofol on pre-synaptic glomus cell chemosensing mechanisms. However, it is of interest that this depression is likely to be independent of TASK (in contrast to the volatile anaesthetics), and also of nicotineric and GABA-ergic mechanisms.

However, in Chapter 5 I have established that the intravenous anaesthetic dexmedetomidine preserves the cellular response to hypoxia, and also at clinically relevant doses in animals AHVR is preserved. This lends weight to the use of dexmedetomidine as an agent of choice in situations where preservation of hypoxic sensitivity is critical, e.g. procedural sedation or critical care environments (He et al., 2014).

In chapter 6, I present evidence that pharmacologically blocking TASK 1 or TASK 3 using novel and specific TASK channel antagonists is sufficient to cause glomus cell excitation, the primary step of the hypoxic chemoreflex signalling cascade. Not only do they excite glomus cells, but they augment the cellular response to mild hypoxia, turning up the chemoreceptor gain. In the presence of isoflurane, antagonising TASK 1 restores cellular hypoxia sensing. These compounds are therefore good candidates for stimulating breathing, and reversing the ventilatory depression caused by anaesthetics.

### **Technical challenges**

The interbreeding of TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> to create double knockout animals took far longer than expected, and litter sizes were also initially poor. However, it was important to recapitulate this genotype as when embarking on this program of work it was expected that the TASK 1<sup>-/-</sup>/TASK 3<sup>-/-</sup> animals would exhibit a normal AHVR (Mulkey et al., 2007). This was demonstrated not to be the case, and although the insights into oxygen and CO<sub>2</sub> sensing have been invaluable, the effect of volatile agents on breathing in double knockouts could not be studied due to this more severe (and unexpected) phenotype.

The ventilatory behaviour of C57BL6 mice, on which background many transgenic models are maintained, is inherently unstable. Some have gone as far as stating that this genotype could be used as a model for sleep-disordered breathing (Yamauchi et al., 2010). Some of these characteristics I exploited (Appendix A), though it is without doubt that a stable breathing phenotype as a background would improve data fidelity. To this end transgenic rats may prove to be this platform.

Cell yields from a tiny organ such as the carotid body are inherently variable. There is a reliance on skill to excise the organ, as much as there is variability with age in the amount connective tissue that impedes isolating glomus cells. The power of this technique to assess native cells and channels is not in doubt, but it would be ideal to study the anaesthetic effects on TASK and oxygen sensing in a more reliable model (e.g. *Xenopus* oocytes) even if only to establish the preliminary mechanisms, before working with native cells.

### **Future work**

#### **By what mechanism does propofol perturb the hypoxic response in the glomus cell?**

Although I have established that propofol depresses glomus cell chemoexcitation, the mechanism by which this occurs is still unclear. A thorough analysis of the effect of propofol on the glomus cell membrane using voltage clamping is critical to understanding this process. By controlling membrane potential, it would be possible to definitively assess the influence of propofol on  $Ca^{2+}_v$  entry into glomus cells. Emerging evidence of the influence of T-type calcium channels (Makarenko et al., 2015) and sodium conductance (Kang et al., 2014) on hypoxic chemosensation suggests a more complex depolarisation cascade than previously thought. The electron transport chain could be perturbed at any level by anaesthetics (Agarwal et al., 2014), and it is established that mitochondria play an important role in hypoxic sensing (Buckler and Turner, 2013). Therefore, it would also be necessary to assess the effects of propofol on the mitochondria of glomus cells, using a combination of NADH auto-fluorescence (which could implicate Complex I) and mitochondrial membrane potential analysis (for instance using rhodamine 123 fluorescence

quenching to study proton flow into the mitochondrial matrix). Should an effect be demonstrated at this level, it would suggest that oxygen sensing is delimited upstream of the cell membrane.

**Do sedative (or sub-sedative analgesic) doses of dexmedetomidine preserve chemoreflexes in humans?**

In order to prove clinical utility, it is essential that a controlled study (using dynamic end-tidal forcing) in humans of the effects of dexmedetomidine is conducted.. Although sedative doses are likely to depress breathing through central nervous system mechanisms (Danielson et al., 2014), if the peripheral chemoreception is intact it could be expected that dexmedetomidine would at least partially preserve the whole reflex loop. At sub-sedative doses, dexmedetomidine may have use as an analgesic, so for this reason it should also be assessed at these concentrations. The vast majority of surgical patients receive an opioid peri-operatively, which are universally depressant of ventilation. Low-dose dexmedetomidine infusions could be opioid-sparing and safe, if it proved that this drug spares the chemoreflex.

**What are the effects of antagonising TASK 1 and TASK 3 in intact animals?**

To date, the only reports of using the compounds A1899 and PK-THPP are from anaesthetised animals (Cotten, 2013) and cellular studies (Streit et al., 2011; Coburn et al., 2012; Rinné et al., 2015). This introduces a significant confounder to understanding the mechanisms by which they work. Using unrestrained plethysmography, it would be possible to dissect the anaesthetic effects on ventilation from the drug effects on ventilatory reflexes. It would then be possible to probe in a more detailed fashion whether these compounds are

## *Chapter 7 – Discussion and Conclusions*

plausible as not only ventilatory stimulants, but also whether they reverse anaesthetic AHVR depression (with or without an effect on hypnosis).

In summary, this thesis has strengthened the current literature on the mechanisms by which anaesthetics influence hypoxic chemosensation in both glomus cells and intact animals. The value of these results will include a new interpretation of work that has gone before (the effect of propofol at the carotid body and the importance of both TASK 1 and TASK 3 to hypoxic chemosensitivity) and in establishing new tools to excite glomus cells (the TASK antagonists A1899 and PK-THPP). Future investigations will benefit from the findings and questions posed by this research.

**Appendix A**

**FURTHER ANALYSIS OF BREATHING PATTERNS IN MICE**

## **Overview**

Given the findings that both single channel TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals (Chapter 3) had a deficit in AHVR, in a post-hoc analysis, I sought to further describe their ventilatory patterns.

Following a period of hypoxia, breathing during reoxygenation is characterised by complex changes in breathing frequency and tidal volume. The best described phenomenon is that of short term potentiation (STP) of  $V_E$ , where  $V_E$  remains greater than the euoxic values before hypoxia for a period of minutes. STP has been demonstrated in rats (Hayashi et al., 1993), cats (Wagner and Eldridge, 1991) and humans (Dahan et al., 1995). However, in mice it is possible that strain differences are important and influence post-hypoxic breathing behaviour (Han et al., 2001). Whereas C57BL6 animals were shown to demonstrate STP, A/J animals did not.

Another feature described in reoxygenation is post hypoxic frequency variation (PHFV). This describes the apparent stochastic nature of breath-to-breath intervals after a period of hypoxia, and may well be a pathophysiological component of breathing disorders such as obstructive sleep apnea (White, 2005). C57BL6 mice exhibit PHFV making them a model for human sleep apnea syndromes (Han et al., 2001; Han et al., 2002).

As I found the TASK 1<sup>-/-</sup>, and TASK 3<sup>-/-</sup> animals to show an abnormal ventilatory response to hypoxia I considered whether they exhibit similar features such as STP or PHFV as the background C57BL6 strain (Gonsenhauser et al., 2004; Getsy et al., 2014). This may give some insight into which part of the respiratory

controller is deranged, as post hypoxic breathing aberrations are thought to be a function of central respiratory control, and independent of peripheral and central chemoreception.

## **Methods**

Flow-time relationships for 8 animals of each strain (C57BL6, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup>) during unrestrained plethysmography in isocapnic and poikilocapnic hypoxia were sampled at 100 Hz and extracted from Finepointe software (Buxco).

Data captured 90 seconds of immediately pre-hypoxia and 90 seconds three minutes after reoxygenation (into air). Average values for  $V_E$  and frequency calculated and analysed for each genotype, in both isocapnic and poikilocapnic hypoxia. Post hypoxic breathing patterns were compared to pre-hypoxic regularity using quantitative analysis of Poincaré plots (See Chapter 2 – General Methods). Return plots (breath-to-breath intervals, plotted against the previous interval) were plotted for each animal, and the standard deviation from the mean compared using Labchart (AD Instruments). Values for SD1 (standard deviation perpendicular to line of unity) SD2 (standard deviation along line of unity) were quantified and compared for each genotype. Data are presented as mean  $\pm$  SEM unless otherwise stated and analysed using repeated measures ANOVA.

## **Results**

### **Post hypoxic frequency**

As anticipated, there was a significant effect of genotype on ventilatory frequency change post hypoxia ( $p < 0.001$ ). Post-hoc analysis revealed this to be

due to a significant decline in frequency in TASK 1<sup>-/-</sup> after isocapnic hypoxia ( $364 \pm 19 \text{ min}^{-1}$  vs  $321 \pm 30 \text{ min}^{-1}$ ,  $p=0.049$ , Figure A.3), but not in other strains.

### **Post hypoxic $V_E$**

There was a small increase in ventilation post poikilocapnic hypoxia across all genotypes, though this did not reach statistical significance (Figure A.2A,  $p=0.192$ ). However, in isocapnic hypoxia, TASK 3<sup>-/-</sup> animals exhibited a significant decline in  $V_E$  post-hypoxia ( $5.7 \pm 0.5$  vs  $3.9 \pm 0.3 \text{ ml}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ ,  $p < 0.001$ , Figure A.1D).

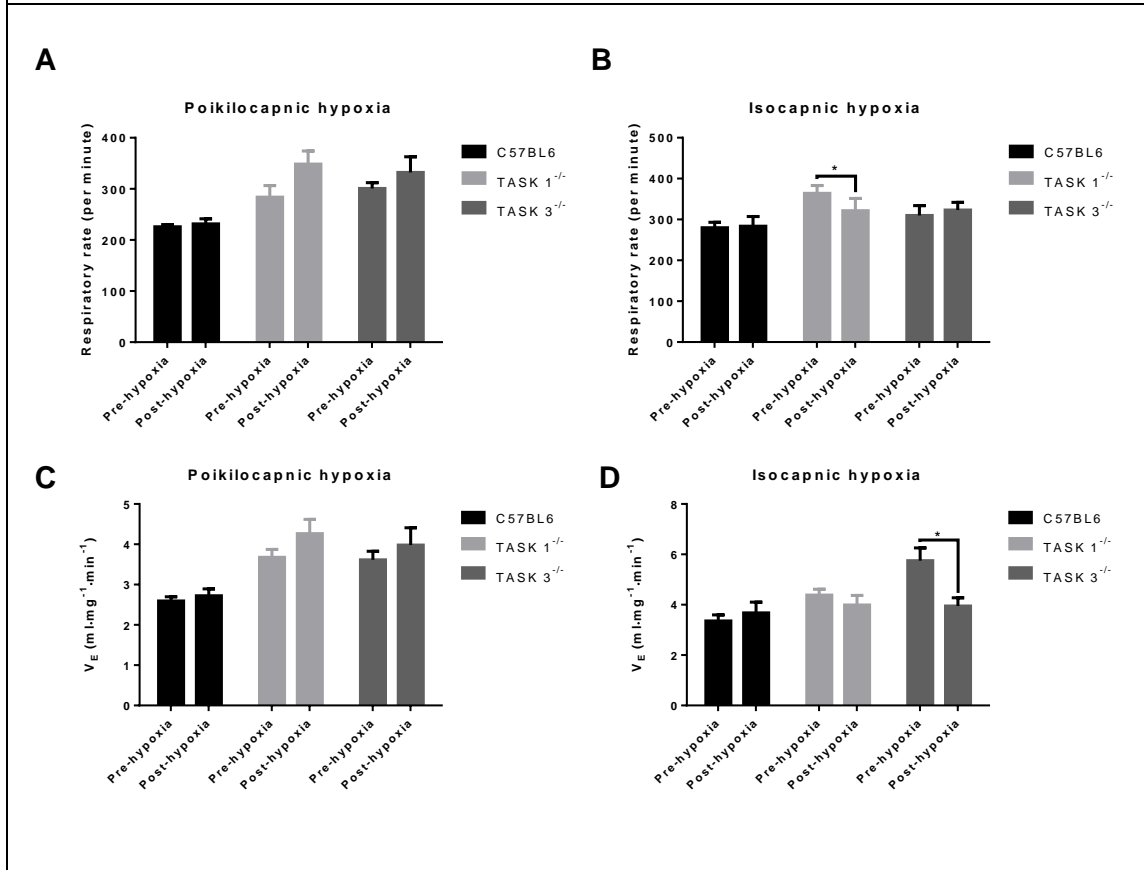
### **Post hypoxic frequency variation**

Figure A.2 and Figure A.3 show representative Poincaré (return) plots for an animal of each genotype in poikilocapnic and isocapnic hypoxia respectively.

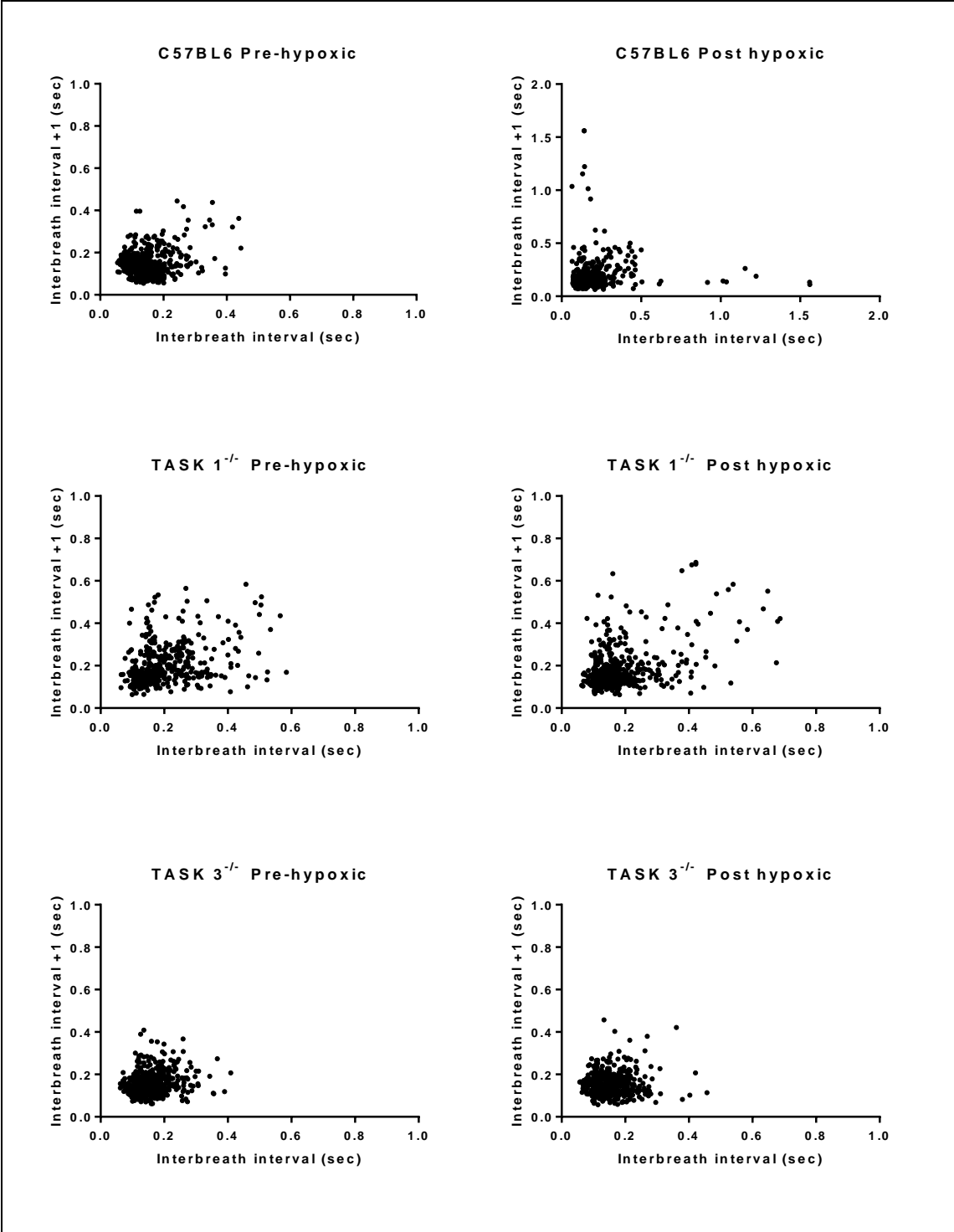
There was significance for the factor “genotype” ( $p=0.003$ ), but not “genotype\*hypoxia” ( $p=0.301$ ), due to a consistent increase in SD1 in both hypoxic conditions in the C57BL6 animals, but in neither TASK 1<sup>-/-</sup> nor TASK 3<sup>-/-</sup> (Table A.1 and Table A.2).

There was no significant interaction for SD2 in “genotype”, nor between the type of hypoxic stimulus.

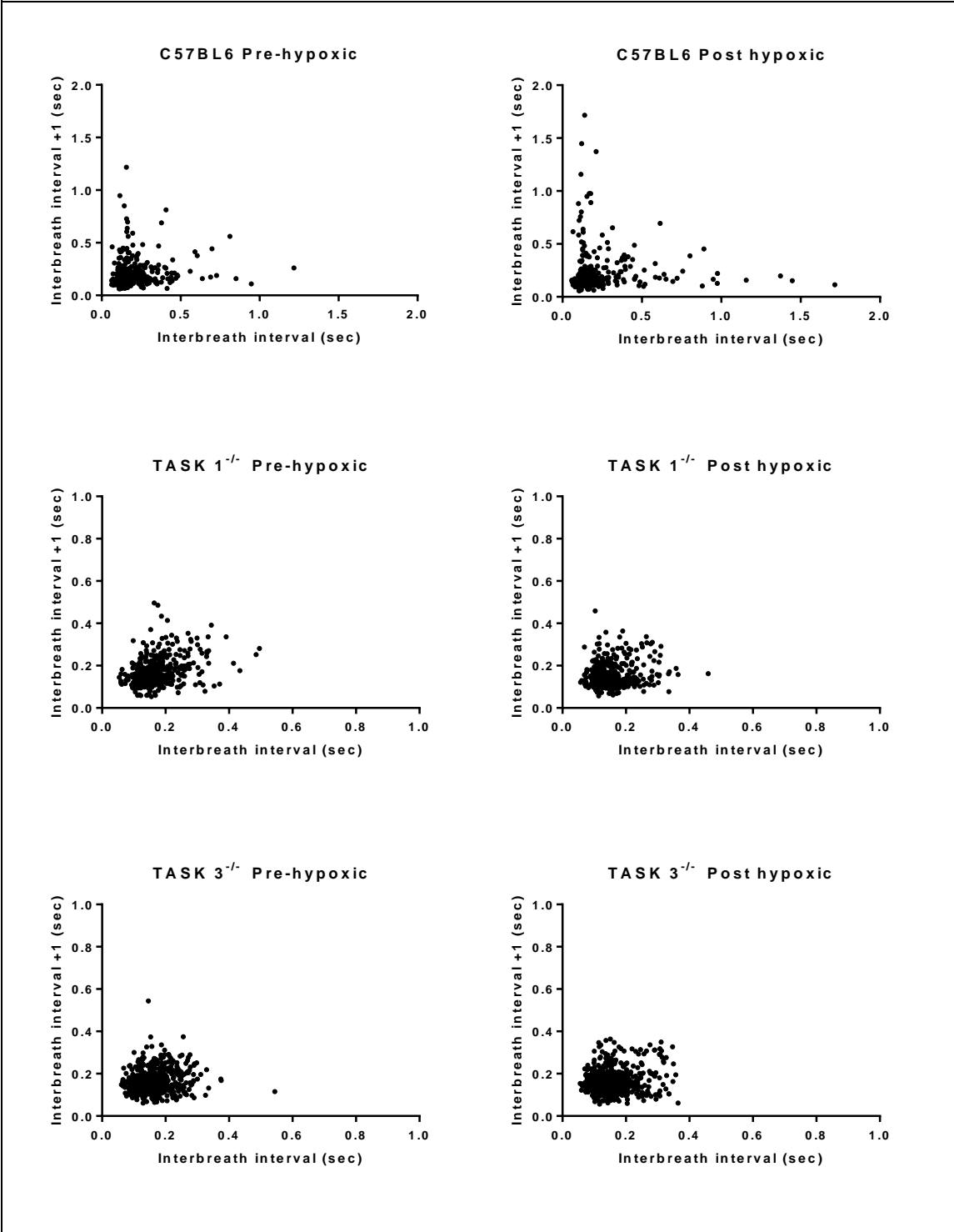
**Figure A.1 A-B** Pre- and post-hypoxic frequency variation for poikilocapnic and isocapnic hypoxia respectively. **C-D**. Pre- and post-hypoxic minute ventilation variation for poikilocapnic and isocapnic hypoxia respectively. The only significant interactions are post-hypoxic frequency decline in TASK 1<sup>-/-</sup> animals (**B**) and post-hypoxic ventilatory decline in TASK 3<sup>-/-</sup> animals. C57BL6 genotype animals demonstrated similar average minute ventilatory and frequency in both poikilocapnic and isocapnic hypoxia.



**Figure A.2** Representative Poincaré plots for post poikilcapnic hypoxic breathing in C57BL6, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup>. It can be seen that C57BL6 develop outliers perpendicular to both axes, representing highly irregular breathing in the post hypoxic period. TASK 1<sup>-/-</sup> appears similar to C57BL6 in the pre-hypoxic period, with TASK 3<sup>-/-</sup> exhibiting more regular breathing in this phase. TASK 1<sup>-/-</sup> has a similar pattern, pre- and post-hypoxia, whereas TASK 3<sup>-/-</sup> appears to become more irregular (though this was not statistically significant).



**Figure A.3** Representative Poincaré plots for post isocapnic hypoxic breathing in C57BL6, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup>. As in **Figure A.2**, post hypoxia, C57BL6 animals have a wider splay of breath-to-breath intervals, whereas TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals maintain a similar pattern of regular breathing as in prior euoxic breathing.



Appendix A Further analysis of breathing patterns in mice

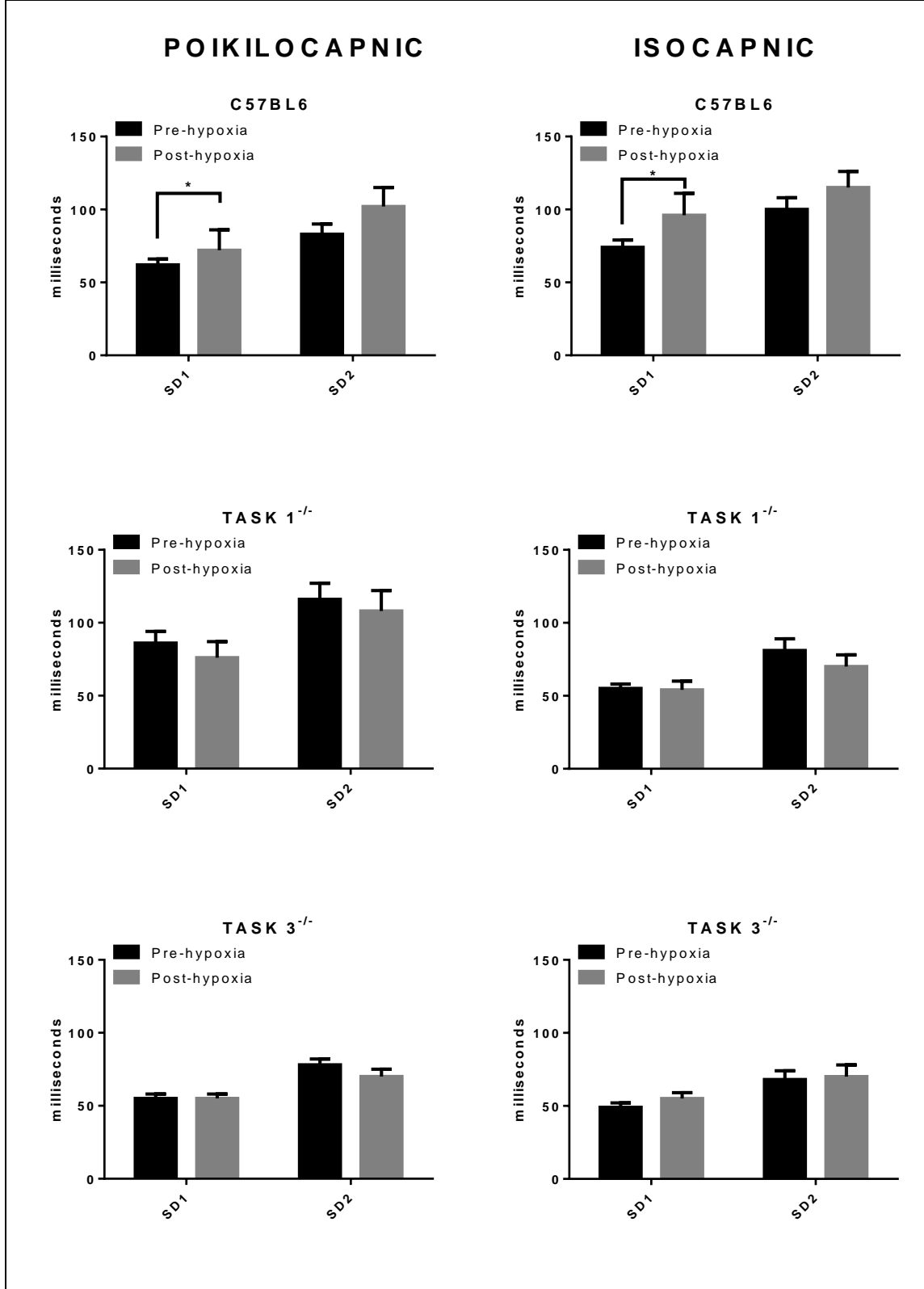
**Table A.1** Quantitative analysis of Poincaré plots for C57BL6, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals pre and post poikilocapnic hypoxia. Values are in milliseconds ± SEM.

	C57BL6		TASK 1 <sup>-/-</sup>		TASK 3 <sup>-/-</sup>	
	Pre	Post	Pre	Post	Pre	Post
SD1	62 ± 4	72 ± 14	86 ± 8	76 ± 11	55 ± 3	55 ± 3
SD2	83 ± 7	102 ± 13	116 ± 11	108 ± 14	78 ± 4	70 ± 5

**Table A.2** Quantitative analysis of Poincaré plots for C57BL6, TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals pre and post isocapnic hypoxia. Values are in milliseconds ± SEM.

	C57BL6		TASK 1 <sup>-/-</sup>		TASK 3 <sup>-/-</sup>	
	Pre	Post	Pre	Post	Pre	Post
SD1	74 ± 5	96 ± 15	55 ± 3	54 ± 6	49 ± 3	55 ± 4
SD2	100 ± 8	115 ± 11	81 ± 8	70 ± 8	68 ± 6	70 ± 8

**Figure A.4** Quantitative representation of the relationship between SD1 and SD2 pre- and post-hypoxia for each genotype. Both poikilocapnic and isocapnic are presented. As seen in **Figure A.2** and **Figure A.3**, the variation in breath-to-breath interval (quantified here as SD1) is greater only in the C57BL6 genotype.



## **Discussion**

This short analysis supports the assertions made in Chapter 3, that TASK 1<sup>-/-</sup> and TASK 3<sup>-/-</sup> animals have significant differences in ventilatory control when compared with C57BL6.

Whereas C57BL6 display significant post-hypoxic frequency variation, neither knockout genotype display this phenomenon, possibly indicating a difference in their underlying central ventilatory control. However, I do not know which is “normal” nor what part of the controller is deranged to produce such a response.

In contrast to previous reports (Han et al., 2001; Han et al., 2002), I was unable to demonstrate short term potentiation of  $V_E$  in C57BL6 animals, nor post-hypoxic frequency decline in this strain. However, the observation of PHFV in TASK 1<sup>-/-</sup> animals supports the hypothesis that this is a genetically mediated feature of breathing control (Tankersley et al., 1994). This could indicate that in addition to a deficit in peripheral chemosensation (Chapter 3), TASK 1<sup>-/-</sup> animals also have significant perturbation of central respiratory rhythm control mechanisms (when compared with C57BL6).

**Chapter 9:**

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### Concluding Figure

In the 1920s, Fernando de Castro produced some of the seminal work on carotid body anatomy. As part of the Ramón y Cajal school of anatomists, he laid the foundations for much of the work produced in this field since. His work was not only meticulous in its detail but the published illustrations are beautiful. Clearly illustrated here are the characteristic “glomi” of Type I cells, with their dense-cored vesicles, that have become so familiar to me over the course of this program of work.

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