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TOPICAL REVIEW

Redefining the components of central CO_2 chemosensitivity – towards a better understanding of mechanism

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Abstract The field of CO_2 chemosensitivity has developed considerably in recent years. There has been a mounting number of competing nuclei proposed as chemosensitive along with an ever increasing list of potential chemosensory transducing molecules. Is it really possible that all of these areas and candidate molecules are involved in the detection of chemosensory stimuli? How do we discriminate rigorously between molecules that are chemosensory transducers at the head of a physiological reflex *versus* those that just happen to display sensitivity to a chemosensory stimulus? Equally, how do we differentiate between nuclei that have a primary chemosensory function, *versus* those that are relays in the pathway? We have approached these questions by proposing rigorous definitions for the different components of the chemosensory reflex, going from the salient molecules and ions, through the components of transduction to the identity of chemosensitive cells and chemosensitive nuclei. Our definitions include practical and rigorous experimental tests that can be used to establish the identity of these components. We begin by describing the need for central CO_2 chemosensitivity and the problems that the field has faced. By comparing chemosensory mechanisms to those in the visual system we suggest stricter definitions for the components of the chemosensory pathway. We then, considering these definitions, re-evaluate current knowledge of chemosensory transduction, and propose the 'multiple salient signal hypothesis' as a framework for understanding the multiplicity of transduction mechanisms and brain areas seemingly involved in chemosensitivity.

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Introduction

Several recent reviews summarize current knowledge of chemosensory reflexes and mechanisms (Chernov *et al.* 2010; Darnall, 2010; Dean & Putnam, 2010; Duffin, 2010; Erlichman *et al.* 2010; Gargaglioni *et al.* 2010; Goridis & Brunet, 2010; Guyenet & Mulkey, 2010; Guyenet *et al.* 2010; Hodges & Richerson, 2010; Kc & Martin, 2010; Kuwaki *et al.* 2010; Milsom, 2010; Nattie & Forster, 2010; Patwari *et al.* 2010; Smith *et al.* 2010; Nattie, 2011; Ray *et al.* 2011). Our purpose is slightly different: to re-examine

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the current assumptions and arrive at new approaches to analyse and understand the apparent complexity underlying chemosensory mechanisms.

The importance of chemosensitivity for breathing

To maintain homeostasis in the face of ever changing circumstances that arise from activity and environmental conditions, physiological systems are subject to numerous chemosensory feedback mechanisms. Respiratory chemosensitivity is the ability of the brain to detect changes in CO_2 and alter physiological systems to regulate its levels within tightly controlled parameters. CO_2 sensitive cells are predominantly within the brain (Feldman *et al.* 2003), though a smaller contribution comes from the carotid bodies (Peers & Buckler, 1995). Mean arterial P_{CO_2} (Pa_{CO_2}) does not change with exercise (Forster *et al.* 1986) and remains constant from well below to above sea level, until prolonged periods are spent there or extreme altitudes (>2500 ft (762 m)) are reached (Haldane & Priestley, 1905; Catron *et al.* 2006). Pa_{CO_2} is even regulated at the expense of P_{O_2} (Haldane & Priestley, 1905), and an increase in Pa_{CO_2} by just 1 mmHg causes a 20–30% increase in ventilation (Feldman *et al.* 2003) and increasing inspired CO_2 to 4% increases ventilation by 177% (Haldane & Priestley, 1905). The primacy of CO_2 in controlling respiration is evident from the earliest land dwelling animals, amphibians (Tayor *et al.* 2003), suggesting a high degree of evolutionary conservation in this vital physiological reflex. Recently, however, the idea that basal levels of P_{CO_2} drive normal breathing (originating from Haldane & Priestley, 1905) has been called into question, as genetically altered mice apparently lacking CO_2 chemosensitivity can still breathe and survive to adulthood (Ramanantsoa *et al.* 2011).

Other physiological chemosensitive reflexes

Hypercapnia can become a life threatening physiological condition through dangerous alterations in blood pH and the increased chance of asphyxiation. Thus, several protective physiological reflexes and behaviours exist. The cardiovascular response, hypertension and bradycardia (Oikawa *et al.* 2005), may allow better alveolar gas exchange or counteract the effects of CO_2 on vasodilatation and autoregulated organs (i.e. the brain and kidney). Renal output alters when blood CO_2 is high, which conserves bicarbonate ions that buffer pH changes caused by hypercapnia. Arousal from sleep and increased vigilance are well known responses to elevated CO_2 (Haxhiu *et al.* 2001; Johnson *et al.* 2005; Williams *et al.* 2007; Buchanan & Richerson, 2010) warning us that either ventilation is inadequate or a dangerous build up of atmospheric CO_2 has occurred. A lack of this response is associated with dysfunctions in chemosensation that lead to death (e.g. sudden infant death syndrome). The association of fear/anxiety to hypercapnia (Papp *et al.* 1993) causes us to leave, or not enter, areas where CO_2 levels exceed survivable limits.

The problems with chemoreception

Comparison to the visual system. To provide a more encompassing framework to consider CO_2 chemosensory processes, we reflect on a better understood sensory system. In the visual system, there is an easily identifiable sensory organ (the eye), which contains an obvious light-detecting neural nucleus, the retina. The retinal photoreceptors contain the entire transduction process that converts photons of light into the release of a primary transmitter that communicates with second order neurons. The photoreceptors, which are directly sensitive to different aspects of the incident light stimulus, are at the head of parallel pathways of visual information, the processing and abstraction of which begins within the retina itself. These pathways are kept separate and recombined in complex ways through convergence, to abstract further features of the visual input.

Even in a system with a recognisable sensory organ and primary neural nucleus, identification of the primary photosensitive cells has not been trivial. Within the retinal ganglion cell population, light-sensitive cells are mixed in with (non-light sensitive) follower cells (Foster *et al.* 1991; Foster & Bellingham, 2004; Hankins *et al.* 2008). The discovery of light-sensitive ganglion cells and an understanding of their function, was only achieved recently through identification of the opsin-based transduction process within them. Interestingly, the removal of the opsins that govern vision did not alter the circadian clock; instead circadian input depends on transduction mediated by melanopsin. Thus, in the eye there are parallel sensory systems that are initiated by the same stimulus, but with distinct transducers, which are juxtaposed but subserve different behaviours.

Consideration of the visual system thus provides a useful analogy when thinking about CO_2 chemosensitivity. In this system the central chemosensing organ is the brain itself and easily identifiable nuclei and chemosensory pathways do not exist. Several central chemosensing 'retinas' have been proposed, each using a different signal transduction pathway and releasing distinct neurotransmitters. Like light sensitive ganglion cells of the retina, primary chemosensitive cells in the CNS are mixed in with other cells and are not readily recognizable.

The key message from the retina is that identification of the transducing molecules is an integral part of the definition of primary sensory cells. These primary chemosensitive cells may be organised into chemosensory nuclei

and multiple such nuclei may subserve different functional systems. Furthermore within the chemosensitive pathways the second or higher order neurons will respond to chemosensitive signals but may not themselves be intrinsically chemosensitive – just as neurons within the visual pathway respond to light impinging on the retina, and are thus light sensitive, without being intrinsically so. Different aspects of the chemosensitive signal could be handled by parallel pathways that ultimately converge and the chemosensitive nuclei and pathways are likely to be organised into integrated systems (Fig. 1).

Maturation of chemosensory responses – practical problems for investigators. Central chemosensory responses begin on embryonic day (E14.5) before the onset of fetal breathing movements (Eugenin *et al.* 2006; Thoby-Brisson *et al.* 2009). In neonatal preparations hypercapnia stimulates ventilation without eliciting metabolic responses (changes in O_2 consumption, thermoregulation or metabolic rate) that modify demand for ventilation (Mortola & Lanthier, 1996). They increase ventilation via changes in tidal volume prior to postnatal day P3 (Stunden *et al.* 2001), and frequency thereafter (Wickström *et al.* 2002). The level of respiratory chemosensitivity varies with age (Putnam *et al.* 2005), being high between P1 and P5, waning to a nadir at P8, and rising again until the adult response is reached by P21 (Stunden *et al.* 2001; Putnam *et al.* 2005). The decrease in the hypercapnic ventilatory response corresponds to a reduction in hypercapnia-induced c-fos staining, occurring after P6 (Wickström *et al.* 1999). This may arise because chemoreceptors are closer to the surface, where they are more able to detect fluctuations in blood chemistry, in neonates than adults (Forster *et al.* 1997). Compensation after P8 may be due to the migration of dendrites to the surface to re-establish their association with blood vessels. It appears more likely though that areas important in neonatal chemosensitivity decrease in importance with age between P1 and P8, and areas important in adult chemosensitivity develop around P12. Hence there is a hiatus between when the neonatal areas switch off and the adult regions switch on. Chemosensitivity increases after this point as the adult mechanisms mature. Once adult chemosensitivity is established it appears to remain constant throughout life, at least in humans (Browne *et al.* 2003).

This has very important consequences for both experimental design and interpretation of data. Several

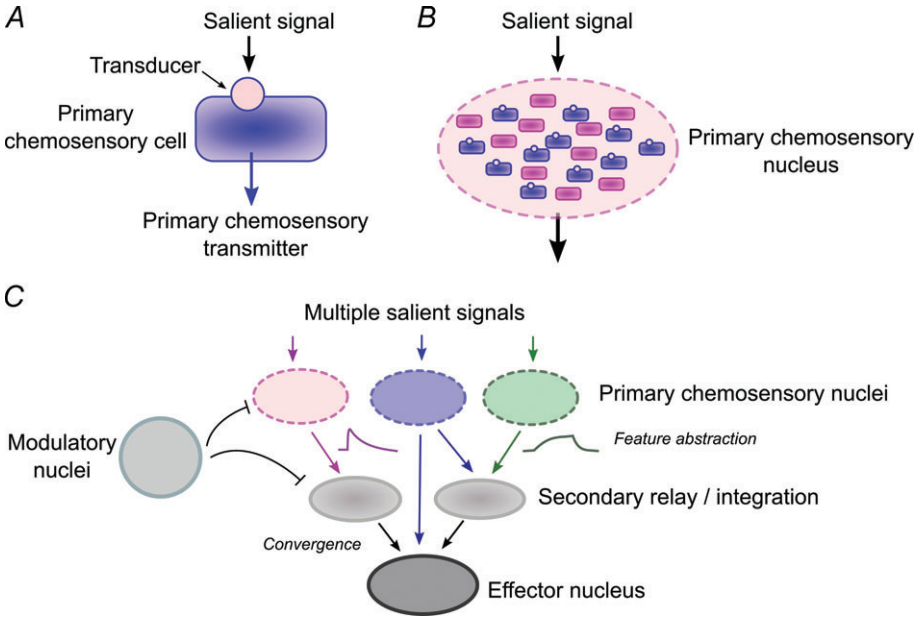

Figure 1. Conceptual organization of chemosensory systems
A, a primary chemosensory cell is defined by possession of a molecular transducer for one of the salient chemosensory signals (pH_i, pH_e, CO_2, HCO_3^-). The link between the transducer molecule and the behavioural reflex must have been established by genetic or pharmacological manipulation. *B*, a primary chemosensory nucleus contains primary chemosensory cells (blue rectangles). These may be intermingled and synaptically connected to non-chemosensitive follower cells (red rectangles). *C*, a chemosensory system may comprise more than one primary chemosensory nucleus possibly detecting different salient chemosensory signals and abstracting different dynamic features of the stimulus (coloured lines are schematic of different types of post-stimulus time firing histograms for the chemosensory cells). These inputs may pass either directly to the effector nucleus or through secondary relays where integration of the signals may occur. In addition, there may be convergent modulatory nuclei that can alter the responsiveness and sensitivity of the system.

different preparations are used to study chemoreception, each confined to a specific developmental period. The brainstem spinal cord (Suzue, 1984) and acute rhythmic transverse slice (Smith *et al.* 1991) preparations are limited to the neonates. The *in situ* preparation (Paton, 1996) can be used for study of mechanisms in juvenile to adult rats. Whole animal experiments are usually limited to the adult. Thus when performing any experiment it is important to use the correct preparation for the appropriate nucleus; it is counterproductive to use neonatal preparations to study nuclei that are important in adulthood and vice versa. The use of inappropriate preparations has led to much conflicting data in the field of chemoreception. It is important therefore to consider the nucleus and the developmental period of the tissue at the time of experimentation when interpreting data.

Molecules and terminology. Once in the extracellular fluid, CO_2 combines with water (facilitated by carbonic anhydrase (CA)) to produce bicarbonate ions and protons (Fig. 2), resulting in three possible species that could act as chemosensory signals, CO_2, HCO_3^- and H^+. For many years the popular consensus was CO_2 is detected entirely through its proxy, pH (Loeschcke, 1982). At first the effects of pH were deemed to be extracellular, though now changes in intracellular pH are known to be important. This concentration on pH has led to the frequent use of imprecise language to describe chemosensitivity – the term 'CO_2 chemosensitivity' commonly refers to detection of pH. Although the evidence does not rigorously exclude other potential signalling molecules, the involvement of direct detection of CO_2 or HCO_3^- has only recently gained attention. Careful simultaneous adjustment of P_{CO_2}, [HCO_3^-] and pH, has shown that different chemosensitive cells respond to different stimuli (Filosa *et al.* 2002; Wang *et al.* 2002; Mulkey *et al.* 2004; Hartzler *et al.* 2008; Huckstepp *et al.* 2010*a*,*b*). Given that molecular transducers for detection of CO_2 and HCO_3^- have emerged, raising the idea of different forms of chemosensitivity, it is important to adjust the language of the field accordingly, i.e. pH chemosensitivity, bicarbonate chemosensitivity and CO_2 chemosensitivity. Even more importantly, the analysis of mechanisms should identify the relevant signalling species at each of the sites involved in chemosensitive reflexes.

As CO_2, HCO_3^- and H^+ are intimately linked via CA it is very difficult to isolate which of them acts as the *salient* signalling molecule in any physiological reflex. CA in effect plays a key role in creating multiple physiological signalling molecules, and is active in many chemosensory nuclei (Coates *et al.* 1993). There are several isoforms of CA with differing properties: CA IV requires high levels of CO_2 ∼20–30% (Chandrashekar *et al.* 2009) whereas CA II only requires ∼1% (Hu *et al.* 2007); some can be modulated by phosphorylation (Narumi & Miyamoto, 1974); and at least one subtype binds to and modulates acid transporters (Li *et al.* 2002, 2006*b*), thus altering intracellular pH by a second and independent mechanism. Therefore, the expression of different CA subtypes may play an important role in the chemosensitivity of different nuclei.

$$\mathbf{CO_2} + H_2O \overset{CA}{\rightleftharpoons} H_2CO_3 \rightleftharpoons \mathbf{HCO_3^-} + \mathbf{H^+}$$

Figure 2. The conversion of carbon dioxide to hydrogen ions under the influence of carbonic anhydrase (CA)
Possible salient chemosensory signals are highlighted in black.

A further practical difficulty arises from studies that substitute the physiological CO_2/HCO_3^- buffering system with Hepes buffered salines (Mulkey *et al.* 2004; Huang *et al.* 2006; Dergacheva *et al.* 2011). This substitution has several potentially serious drawbacks: it alters both extracellular and intracellular buffering capacity (as CO_2 readily crosses membranes); it removes the influence of carbonic anhydrase; the lowered intracellular concentration of HCO_3^- will affect the activation of soluble adenylyl cyclase and a number of transport systems for HCO_3^-; and Hepes and other organic aminosulphonated compounds can inhibit gap junctions and hemichannels (Bevans & Harris, 1999; Tao & Harris, 2004). At least one gap junction hemichannel protein, connexin26 (Cx26), is important in chemosensory transduction (Huckstepp *et al.* 2010*a*,*b*). Therefore considerable caution when interpreting these data is required, as it cannot necessarily be applied to the *in vivo* system. An example of this has been reported in a recent study where the presence of the CO_2/HCO_3^- was essential to see the involvement of ATP in the responses of chemosensory neurons (Wenker *et al.* 2010).

Anaesthesia, conscious animals and chemosensitivity. Anaesthetics can depress the firing of neurons and interfere with components of chemosensitivity (Knill *et al.* 1983; Koh & Severinghaus, 1990; Nattie, 2001), potentially altering the balance of mechanisms in whole animal responsiveness to CO_2. It is very important to also establish and test mechanisms of chemosensitivity, initially posited from reduced *in vitro* preparations and anaesthetised animals, in awake freely behaving animals.

Towards a solution – definitions

Chemosensory transducer. As the mechanism of transduction of a chemosensory signal is crucial in classifying intrinsic chemosensory cells and primary chemosensory transmitters (Fig. 1), we need to define rigorous criteria for the identification of chemosensory transduction molecules. This is not straightforward as it is likely that

many molecules have *incidental* sensitivity to various aspects of chemosensory stimuli, i.e. while a molecule may be sensitive to pH, this may not play a role in the intrinsic chemosensitivity of a given cell or nucleus.

We define a chemosensory transducer as a molecule that responds to fluctuations of a salient chemosensory stimulus (pH (either internal or external), P_{CO_2}, HCO_3^-) in a manner that results in significant changes in cellular excitability or cell–cell signalling. Critically, genetic deletion and/or selective blockade of this molecule must reduce or ablate the behavioural chemosensory reflex in conscious animals, thus showing that the molecule is *necessary* to endow a cell/nucleus with intrinsic chemosensitivity. Expression of the relevant molecule should be *sufficient* to endow a (previously unresponsive) cell with responsiveness to the salient chemosensory signal. Demonstrating the presence of a pH- CO_2- or bicarbonate-sensitive molecule in a cell is insufficient; it is essential to show the presence of this molecule is necessary for the chemosensory reflex.

Intrinsic chemosensitivity. An intrinsically sensitive chemosensory cell (or primary chemosensor) must by definition respond to at least one salient stimulus (CO_2, HCO_3^- or pH (either internal or external)), directly, without dependence on any other cell type in the CNS (Fig. 1). This cell should then communicate, directly or indirectly, with the physiological centres of breathing, arousal, etc. to affect the relevant reflex.

The practical test for intrinsic chemosensitivity has involved demonstration of responses to the relevant chemosensory stimulus following lowering of extracellular Ca^{2+} or in the presence of TTX or antagonist cocktails (Kawai *et al.* 1996; Nattie, 2000; Nattie & Li, 2002; Mulkey *et al.* 2004; Richerson, 2004). As these traditional experimental criteria do not take into account modern knowledge of the multiplicity of mechanisms for cell to cell communication now evident in the CNS (e.g. transmitter release from glial cells does not require extracellular Ca^{2+} and is TTX independent), they are not definitive and leave open the possibility that chemosensitivity is secondary to the release of a transmitter from an (unknown) primary chemosensory cell.

We therefore propose the following definition: an intrinsically chemosensitive cell must contain a molecular transducer that enables it to respond directly to the relevant chemosensory stimulus. The necessity of this molecular transducer for chemosensory responses must be demonstrated by either genetic deletion or selective antagonism. Using this definition, a cell containing a pH/CO_2/bicarbonate sensitive molecule is not a primary chemosensor unless that molecule has been causally linked to at least one of the relevant behavioural chemosensitive reflexes.

Primary chemosensory transmitters. It follows from the above that a primary chemosensory transmitter is that which is released from an intrinsic chemosensory cell (Fig. 1). In a complex pathway involving a sequence of several cells with interposed synapses, it is likely that several transmitters will be released in response to a chemosensory stimulus. Many of these, however, will be as a secondary or downstream consequence. Identification of the transmitter released by the intrinsic chemosensors is non-trivial and will depend upon a series of experimental approaches: directly measuring transmitter release, determining the presence of release mechanisms and genetic and/or pharmacological manipulation of the transduction mechanism/signalling pathway.

Following in the footsteps of Werman (1966), we propose the following criteria necessary to demonstrate the involvement of a neurotransmitter in the chemosensory cascade. The candidate transmitter must be: present in the primary chemosensitive cells; released in response to a chemosensory stimulus prior to activation of the network response; and released onto (or able to reach by diffusion) the effector nuclei that produce the physiological reflex. Finally, selective antagonism of the transmitter must reduce or ablate a physiological response during hypercapnia and application of its agonist must evoke the reflex.

Primary chemosensory nucleus. A primary sensory nucleus must contain intrinsically chemosensitive cells established by the definition given above (i.e. contain a proven molecular transducer). In the absence of these cells, a chemosensory nucleus must be considered as a relay in the chemosensory reflex (Fig. 1).

A reappraisal of the current literature

We now re-examine the evidence for the current molecules, transducers, intrinsic chemosensors and pathways in the light of the definitions outlined above.

Modalities of chemosensing: salient stimuli

A recent comprehensive review of salient stimuli and their targets, in all multicellular organisms regardless of function, has been written (Tresguerres *et al.* 2010). Here we focus specifically on molecules proposed to be involved in the physiological reflexes associated with hypercapnia in mammals.

pH. The primacy of pH in chemoreception is reflected in other fields of physiology, i.e. CO_2 cerebrovascular reactivity and the control of vasodilatation (Brian, 1998; Ainslie & Duffin, 2009). As pH has been largely regarded as the chemosensory signal, much research effort has hinged on studying pH-sensitive ion channels as potential

molecular transducers. So far genetic manipulation of these channels has failed to provide evidence for their involvement in mechanisms of chemoreception. In addition, much prior work show strong indications that pH is not the only chemosensitive signal (Pappenheimer *et al.* 1965; Fencl *et al.* 1966; Loeschcke, 1982; Eldridge *et al.* 1985; Shams, 1985). For example, comparing respiratory output after applying acidic saline to the medulla oblongata or inhalation of additional CO_2 to induce the same pH change showed that the combination of an increase in P_{CO_2} and a decrease in pH was a more powerful stimulant than a decrease in pH alone. To quote Eldridge *et al.*: 'We conclude that the e.c.f. [extracellular fluid] $[H^+]$ does not represent the unique stimulus to the central chemoreceptors. We discuss several alternate mechanisms for the action of CO_2 and $[H^+]$ on central chemoreceptors but none can be considered definitive at the present time' (Eldridge *et al.* 1985). Furthermore combined CO_2 inhalation with intravenous infusion of balancing HCO_3^- to prevent changes of pH measured at the ventral surface of the medulla showed that an increase of PCO_2 at the medullary surface by itself was able to increase respiratory rate (Shams, 1985). This provides strong support for independent effects of pH and PCO_2 on breathing.

CO_2. Recognition of CO_2 as a salient chemosensory signal has been slow to emerge as interactions of CO_2 with potential transducer proteins have been largely overlooked by physiologists. Insects such as mosquitoes are sensitive to CO_2, mediated through G-protein coupled receptors (Jones *et al.* 2007). That CO_2 binds with haemoglobin and how this may apply to direct detection of CO_2 in other contexts has gone unnoticed; here CO_2 reacts to form a carbamate moiety (via a labile covalent bond) on each of the terminal amines of the two α and two β globin chains (Kilmartin & Rossi-Bernard, 1971). In microbial enzymology (Maveyraud *et al.* 2000) and in the fixation of CO_2 (Lundqvist & Schneider, 1991), enzymes are activated by formation of a carbamate moiety on specific lysine side chains. Evidence now suggests specific molecular mechanisms by which CO_2 can interact directly with a number of gap junction hemichannels (Huckstepp *et al.* 2010*a*,*b*), affect inwardly rectifying potassium channels (Huckstepp & Dale, 2011) and bind directly to soluble adenylyl cyclase (sAC) (Townsend *et al.* 2009). There is no longer an absence of a plausible molecular mechanism for the detection of CO_2, providing an impetus for the recognition of CO_2 itself as a salient chemosensory stimulus.

HCO_3^-. A potential molecular detector for HCO_3^- is sAC (Zippin *et al.* 2001; Townsend *et al.* 2009; Tresguerres *et al.* 2010), giving a possible signalling mechanism through the production of cyclic AMP (cAMP); this has been implicated in the chemosensitive response of both the retrotrapezoid nucleus (RTN) (Ritucci *et al.* 2005) and glomus cells of the carotid body (Summers *et al.* 2002; Nunes *et al.* 2009). However, can the fluctuations of HCO_3^- during hypercapnia be sufficient to change the activation of sAC in a functionally significant manner? sAC is most sensitive to bicarbonate ions in the low millimolar range (Chen *et al.* 2000), whereas levels in CSF are usually ∼19 mM and rise by only 3 mM during extended periods of hypercapnia (Kazemi *et al.* 1976), translating into a modest increase in sAC activity (Chen *et al.* 2000). Whether this increase is functionally significant depends on sAC expression levels, cellular cAMP concentration and the extent to which this varies as a result of modulation of sAC by changing HCO_3^- concentrations. That activation of sAC can cause functional changes in cilia of the airway (Schmid *et al.* 2007) implies that it may be. Therefore, we think it now useful to evaluate more systematically sAC as a potential chemosensory transducer.

Transducing molecules

Candidates with genetic evidence. *Acid sensing ion channel (ASIC).* ASICs are expressed in regions of the brain known to be linked to fear associated with hypercapnia, such as the amygdala (Ziemann *et al.* 2009). Cultured neurons that lack the ASIC1a channel no longer respond to alterations in CO_2, whereas wild-type neurons show a strong and graded response to it (Ziemann *et al.* 2009); ASIC1a is therefore sufficient to endow neurons with chemosensitivity. ASIC1a knockout mice have a severely blunted fear response associated with hypercapnia (Ziemann *et al.* 2009), demonstrating that this molecule is necessary for the full hypercapnic response. This provides the first (and only) genetic evidence for a primary chemosensory transducer (Table 1).

Candidates with pharmacological evidence. *Connexin 26 (Cx26).* Cx26 is expressed on the leptomeninges and sub-pial astrocytes, including those at the ventral surface of the medulla that respond to hypercapnia (Huckstepp *et al.* 2010*b*). It responds to changes in P_{CO_2} by opening to release ATP, which subsequently causes adaptive changes in ventilation (Huckstepp *et al.* 2010*a*,*b*). Cx26 seems directly sensitive to CO_2 as it remained chemosensitive in isolated patches of both the inside-out and outside-out configuration (Huckstepp *et al.* 2010*a*). Expression of Cx26 by itself in HeLa cells was sufficient to make them sensitive to CO_2 and to recapitulate CO_2-sensitive ATP release, similar to that seen from the surface of the medulla (Huckstepp *et al.* 2010*b*). Crucially, pharmacological blockade of this channel reduced hypercapnia elicited ATP release *in vitro* and *in vivo*, and reduced the hypercapnia induced adaptive changes in breathing *in vivo* under anaesthesia (Huckstepp *et al.*

2010*b*). Current evidence thus suggests that Cx26 may be both necessary and sufficient for CO_2 chemosensory transduction (Table 1). Nevertheless targeted and conditional genetic manipulation of Cx26 expression (complicated by the lethality of unconditional deletion of this gene) will be required to elucidate the contribution of Cx26 to the full physiological responses to hypercapnia including those in awake animals.

Candidates present with contradictory evidence for involvement. *Tandem-pore acid sensing potassium (TASK) channels.* TASK-1 channels contribute to the pH sensitivity of raphé neurons (Mulkey *et al.* 2007*b*) and maybe the locus coeruleus (LC) (Filosa & Putnam, 2003) and could confer intrinsic chemosensitivity to the preBötzinger Complex (preBötC) (Koizumi *et al.* 2010) and orexinergic neurons of the lateral hypothalamus (Williams *et al.* 2007). Since these acid sensing channels are found in the respiratory centre and in cell groups important for chemosensory function, it is possible their activation may lead to adaptive breathing associated with hypercapnia. Nevertheless, constitutive deletion of the TASK-1 and TASK-3 genes does not alter the hypercapnic ventilatory responses (Mulkey *et al.* 2007*b*). Thus, these channels are either not essential for the central chemosensitive reflex or compensatory mechanisms obscure their role (Mulkey *et al.* 2007*b*; Trapp *et al.* 2008). Only targeted inducible genetic manipulations will discriminate between these possibilities. Nevertheless, TASK-1 is required for correct function of peripheral chemosensors in the carotid body, and contributes to the whole animal response to lower levels of hypercapnia (Trapp *et al.* 2008). Overall there is considerable doubt about the role of TASK channels as central chemosensory transducers (Table 1).

pH sensitive inwardly rectifying potassium (KIR) channels. pH-dependent closure of inwardly rectifying potassium channels could underlie the pH sensitivity of some neurons and glia i.e. KIR 4.1/5.1 heteromer (Xu *et al.* 2000) on sub-pial astrocytes in the RTN (Wenker *et al.* 2010). Here channel closure leads to depolarization of the astrocytes evoking vesicular ATP release (Wenker *et al.* 2010), and pH-dependent ATP release from astrocytes in the RTN can enhance breathing (Gourine *et al.* 2010). However, the link between KIR 4.1/5.1 and the behavioural reflex remains to be definitively established. This possibility was weakened by recent genetic evidence showing KIR5.1$^{-/-}$ mice have an intact central chemosensitive response (Trapp *et al.* 2011), though these experiments may be confounded as these mice have chronic metabolic acidosis (Trapp *et al.* 2011). Additionally, LC neurons derived from these mice show a significant decrease in their pH sensitivity (D'Adamo *et al.* 2011), but this effect on isolated neurons has not been extended *in vivo* or to the behavioural response. Since manipulation of the LC appears to be important in central chemoreception, it is possible that these mice have compensated for the loss of this protein either by upregulation of another subunit, or by plastic changes that place greater emphasis on other nuclei (Trapp *et al.* 2011).

Overall the evidence for involvement of KIR 4.1/5.1 as a chemosensory transducer is inconclusive and will remain so until targeted conditional deletion of these genes has been attempted (Table 1).

pH_i sensitive channel in raphé. The raphé complex probably contains multiple pH-sensitive channels. Whilst TASK-1 may endow neurons with pH_e sensitivity (but see above), raphé neurons have also been shown to be sensitive to pH_i *in vitro* (Wang *et al.* 2002; Ribas-Salgueiro *et al.* 2005) and to some extent *in vivo* (Veasey *et al.* 1995; Ribas-Salgueiro *et al.* 2003; Mulkey *et al.* 2004) suggesting the presence of a further channel sensitive to pH_i. These molecules may not necessarily co-exist in raphé neurons at the same time. Instead, it may be that as chemosensitivity, which changes developmentally (Wang *et al.* 2002), switches from its neonatal to adult form, raphé neurons change their expression of pH sensitive channels to alter their sensitivity to hypercapnia. Nevertheless the status of these unknown channels as chemosensory transducers will remain uncertain until they have been identified and clear pharmacological or genetic evidence links them to an adaptive reflex (Table 1).

Outwardly rectifying potassium channel. Although the chemosensitivity of RTN neurons is preserved in the presence of blockers of excitatory transmission and in TTX (Mulkey *et al.* 2004), these manipulations are not rigorous tests of intrinsic chemosensitivity. Nevertheless RTN neurons may contain a chemosensory transducer of some form and their activation by acidification occurs via reduction of an outwardly rectifying potassium current (Wellner-Kienitz *et al.* 1998; Mulkey *et al.* 2004; Guyenet, 2008), making the channel underlying this current a putative candidate transducer. However until the gene encoding this outward rectifier channel is identified a rigorous test of its role in controlling excitability and adaptive ventilatory behaviour remains out of reach.

Chemosensitive anatomical structures

Putative chemosensitive areas are found close to the surface of the brain with a denser capillary network than the surrounding tissue, usually with their neuronal axon terminals and somata abutted to them (Okada *et al.* 2001; Okada *et al.* 2002); allowing them to sample

Table 1. Hypothesised chemosensory transducers, molecular stimulus, nuclei they are found in and the physiological function they alter

Transducer	Signal	Area	Reflex	Evidence *(in vivo)*
ASIC1a	pH_e	*Amygdala*	Fear/anxiety	G+
Cx26	CO_2	*Sub-pial astrocytes of VMS*	Breathing	P+
TASK-1	pH_e	*PreBötzinger Complex*	Breathing	G−
		Locus coeruleus	Breathing	G−
			Fear/anxiety	corr
		Retrotrapezoid nucleus	Breathing	G−
		Lateral hypothalamus	Arousal/ vigilance	corr
Kir4.1/5.1 possibly other Kirs	pH_i, pH_e	*Locus coeruleus*	Breathing	G−
			Fear/anxiety	corr
		Retrotrapezoid nucleus	Breathing	G−
		Sub-pial astrocytes	Breathing	G−
Kor – not identified	pH_e	*Retrotrapezoid nucleus (neonate)*	Breathing	corr
Unknown	pH_i	*Raphé (adult/neonate)*	Breathing Arousal/vigilance	corr

G+, supporting genetic evidence; G–, contradictory genetic evidence; P+, supporting pharmacological evidence; corr, correlative evidence. pH_i, intracellular pH; pH_e, extracellular pH; Kor, outwardly rectifying potassium channel; Kir, inwardly rectifying potassium channel; ASIC, acid sensing ion channel; TASK, TWIK-related acid-sensitive K^+ channel; Cx26, connexin 26.

blood before it is affected by the intrinsic metabolism of surrounding tissue. These blood vessels are surrounded by a perivascular space (Okada *et al.* 2001), with elongated and labryrinthic invaginations of the sub-arachnoid space which form cisternae (Okada *et al.* 2001), creating a large surface area, with a higher rate of blood delivery, which allows for greater blood gas sampling (Göbel *et al.* 1990).

A large number of putative chemosensory areas have been described in the literature that contribute to several different CO_2-sensitive reflexes. However, a primary chemosensory nucleus must contain primary chemosensitive cells that are directly activated by hypercapnia, defined through their possession of a chemosensory transducer causally linked to the chemosensitive reflex by genetic deletion or pharmacological experimentation. A primary chemosensory area must show direct or indirect connections to a region that is ultimately responsible for altering the output of the reflex (i.e. in adaptive breathing it must ultimately connect to the preBötC). Chemosensitive areas that lack primary chemosensory cells are most likely relay nuclei. It is not necessary that all chemosensitive structures should be arranged in a serial pathway; some regions may be multifunctional and subserve both the chemosensitive and physiological responses (Arendt, 2008).

Though often studied individually, interactions between the leptomeninges, vasculature, astrocytes and neurons within a chemosensory nuclei may mean that it is better to view these components as a coordinated functional unit. In the RTN/pFRG, meningeal cells control the migration of neurons, angiogenesis and glial function, ensuring the precise development and regulation of this chemosensory unit from before birth (Schwarz *et al.* 2004; Thoby-Brisson *et al.* 2009; Grant & Moens, 2010; Rosenstein *et al.* 2010). A similar chemosensory functional unit may also occur in medullary raphé, as the raphé neurons are in close proximity to the marginal glial layer and leptomeninges (Ribas-Salgueiro *et al.* 2005) and are closely associated with arteries (Bradley *et al.* 2002; Severson *et al.* 2003).

Here we review the putative central chemosensitive areas of the brain that have been described and the extent to which they can be considered as primary central chemosensory nuclei. We will also assess at what stage of development they are most important and which reflex they contribute to.

Raphé nuclei

The medullary raphé are found within close proximity to the VLM surface (Ribas-Salgueiro *et al.* 2005), whereas the dorsal raphé are found at the surface of the midbrain (Severson *et al.* 2003). Raphé neurons are highly sensitive to hypercapnia *in vitro* (Wang *et al.* 2002; Severson *et al.* 2003; Ribas-Salgueiro *et al.* 2005) and are connected to other chemosensory sites (Stocker *et al.* 1997;

Ribas-Salgueiro *et al.* 2005; Mulkey *et al.* 2007*b*) and the preBötC (Li *et al.* 2006*a*; Pace *et al.* 2007; Hodges *et al.* 2008; Li & Nattie, 2008*b*; Ptak *et al.* 2009). That they remain chemosensitive isolated in cultures and in an antagonist cocktail supports their intrinsic chemosensitivity (Wang & Richerson, 1999). Nevertheless these are soft criteria.

Their involvement in intact animals has been questioned, as only a small number of raphé neurons exhibit chemosensitivity *in vivo* (Veasey *et al.* 1995; Ribas-Salgueiro *et al.* 2003; Mulkey *et al.* 2004; DePuy *et al.* 2011). Current evidence suggests that these nuclei only play a facilitatory role (Li *et al.* 2006*a*; Mulkey *et al.* 2007*a*; DePuy *et al.* 2011). Medullary raphé neurons are serotonergic and blockade of 5-HT receptors by methysergide (a 5-$HT_{1/2}$ receptor antagonist) in neonates reduces both respiratory rate and c-fos staining in respiratory related areas *in vivo* and *in vitro* (Bodineau *et al.* 2004). Mice with deletion of serotonin transporters have increased levels of 5-HT in their extracellular fluid (ECF), leading to desensitization of their receptors, and a decrease in respiratory responses to CO_2 (Penatti *et al.* 2006; Li & Nattie, 2008*b*). However, until a chemosensory transducer in medullary raphé neurons can be identified and tested, their status as primary chemosensitive neurons will remain open to doubt. Regardless of whether the contribution of the medullary raphé is mainly facilitatory or is indeed as an intrinsic chemosensitive site, inhibition of the medullary raphé significantly reduces the hypercapnic ventilatory response (Taylor *et al.* 2005) and the absence of serotonergic neurons, or silencing of them in genetically modified mice, severely blunts the chemosensitive response (Hodges *et al.* 2008; Ray *et al.* 2011).

Interestingly, loss of serotonergic neurons also causes the loss of the waking response to hypercapnia – a function thought to be mediated by the midbrain raphé (Buchanan & Richerson, 2010). The dorsal raphé of the midbrain are connected to cortical and hypothalamic regions associated with arousal (Vertes, 1991). However, recent evidence questioning the chemosensitivity of the medullary raphé nuclei raises the possibility that the midbrain raphé may also lack intrinsic chemosensitivity. Once again, targeted genetic manipulation of the key chemosensory transducing molecules within the dorsal raphé is required to settle the issue. Until this is achieved we consider the dorsal raphé to be a provisional, rather than an established, primary chemosensory nucleus.

The chemosensitivity of the medullary raphé varies with age: during the first two postnatal weeks there is an increase in neurons responsive to hypercapnia both *in vivo* and in culture (Wang & Richerson, 1999). After the second postnatal week the response remains unchanged (Veasey *et al.* 1995; Okada *et al.* 2002). It would be of interest to see whether their facilitatory role is also age dependent. The raphé complexes may be more crucial to chemosensitivity during adult life than in the neonate; or during the switch from neonatal to adult mechanisms, which leads to a vulnerable period that may explain why defects in this system often leads to sudden infant death syndrome (SIDS) (Duncan *et al.* 2010).

The tuberomammillary nucleus (TMN)

The TMN is located near the ventral surface of the posterior hypothalamus and is the sole source of histamine in CNS. Deletion of histidine decarboxylase (HDC) has been used to study the role of the TMN (Parmentier *et al.* 2002; Anaclet *et al.* 2009). Mice that lack the HDC gene spend more time in paradoxical sleep than their litter mates (Parmentier *et al.* 2002; Anaclet *et al.* 2009) as do mice injected with antagonists of this enzyme (Parmentier *et al.* 2002). Thus, this nucleus is associated with arousal and vigilance (Parmentier *et al.* 2002; Anaclet *et al.* 2009). TMN neurons are excited during hypercapnia (Dillon & Waldrop, 1992) and show increased c-fos immunoreactivity after exposure to elevated CO_2 (Berqin *et al.* 2000; Haxhiu *et al.* 2001; Johnson *et al.* 2005). There is some evidence that activation of neurons in the TMN may increase breathing during hypercapnia (Haxhiu *et al.* 2001); however, a causal link between activity in the TMN and CO_2-dependent arousal has not yet been achieved and there are two other nuclei, the midbrain dorsal raphé (see above) and the lateral hypothalamus, that have also been proposed for this role.

The lateral hypothalamus

Orexinergic neurons of the lateral hypothalamus display increased c-fos immunoreactivity after exposure to hypercapnia (Berqin *et al.* 2000) and increase firing in response to alterations in pH_e (Williams *et al.* 2007). As the lateral hypothalamus is the sole source of orexin, deletion of the prepro-orexin gene (Chemelli *et al.* 1999) is a genetic functional lesion of this area. Although this manipulation clearly demonstrates the importance of orexinergic signalling in maintaining wakefulness (Anaclet *et al.*, 2009; Carter *et al.* 2009), the effect of this lesion on CO_2-dependent arousal has not been examined. Furthermore the link between the putative chemosensory transducer (TASK-1) and CO_2-dependent arousal has not been demonstrated. Thus, the status of this area as a primary pH chemosensor for CO_2-dependent arousal is at best only putative.

Genetic deletion of the prepro-orexin gene blunts respiratory responses to hypercapnia (Deng *et al.* 2007) as do pharmacological antagonists of orexin applied to the medulla (Corcoran *et al.* 2010), although this is primarily during wakefulness (Dias *et al.* 2009, 2010; Li & Nattie, 2010). Orexinergic terminals have been found in close apposition to RTN neurons, which can be excited by exogenous orexin (Lazarenko *et al.* 2011), though it is unknown whether orexin acts to facilitate or initiate

chemosensitive responses in these neurons. In addition, orexin appears to facilitate GABAergic currents on cardiac vagal neurons, which act to induce the bradycardia associated with hypercapnia (Dergacheva *et al.* 2011).

The locus coeruleus (LC)

The LC is located at the ponto-medullary border directly underneath the fourth ventricle in the pons and supplies tonic chemosensory input (Infante *et al.* 2003). Stimulation of the LC causes dopamine and noradrenaline release in the cortex (Devoto *et al.* 2005), which has been linked to arousal and increased vigilance (Devoto *et al.* 2005). It also receives projections from the medial habenula (Li & Ku, 2002), and sends efferent projections to the amygdala, and has been linked to fear and anxiety (Gorman *et al.* 2000; Li & Ku, 2002; Devoto *et al.* 2005). In addition, the LC has been linked to the respiratory response to hypercapnia. Thus, it is poised to affect many of the physiological behaviours associated with hypercapnia.

Hypercapnia causes an elevation of c-fos immunoreactivity in LC neurons (Berqin *et al.* 2000) and focal acidification of the LC increases breathing (Coates *et al.* 1993). Neurons in the LC respond to hypercapnia (Stunden *et al.* 2001; Filosa *et al.* 2002) and to pH changes associated with acetazolamide (AZ) microinjections in the LC (Coates *et al.* 1993). Cultured neurons from the LC remain chemosensitive during pharmacological synaptic blockade and blockade of electrical coupling and in the presence of TTX (Filosa *et al.* 2002; Filosa & Putnam, 2003; Nichols *et al.* 2008). Nevertheless these are not definitive criteria and the status of the LC as a primary chemosensory area can only be considered provisional in the absence of a chemosensory transducer.

LC neurons are catecholaminergic (Yao *et al.* 2000). At the surface of the VLM, noradrenaline (NA) levels increase during hypercapnia (Rentero *et al.* 1997) and metabolic acidosis (Rentero *et al.* 1998). Ablation of noradrenergic neurons, by injections of dopamine β-hydroxylase tagged saporin, leads to a reduction in both the hypercapnic ventilatory response and time spent in the awake state (Li & Nattie, 2006) thereby strengthening the argument that NA, and thus the LC, plays a role in the waking response to hypercapnia in addition to its role in the hypercapnic ventilatory response. Further evidence utilizing 6-hydroxydopamine to lesion LC neurons supports a role for the LC in modulating the ventilatory response to hypercapnia (Biancardi *et al.* 2008).

The LC undergoes maturational changes and there is a dramatic reduction in the number of chemosensitive neurons and in the magnitude of chemosensory responses of single neurons around P10 (Nichols *et al.* 2008). The physiological role of this nucleus in chemosensory transduction may therefore change with development.

Amygdala

The amygdala is the fear control centre of the brain. Importantly neurons in the amygdala contain ASIC1a channels, which have been causally linked (through genetic deletion) to the CO_2-evoked fear reflex, so by our definition these neurons are primary chemosensors (Ziemann *et al.* 2009). Direct stimulation of the amygdala by changes in local pH elicit the fear response and is therefore likely to be one of the areas responsible for fear associated with hypercapnia (Ziemann *et al.* 2009). As the deletion of ASIC1a was global, the possibility of other chemosensitive nuclei contributing to the fear reflex remains open.

The nucleus tractus solitarii (NTS)

The nucleus tractus solitarii is situated superficially in the dorsal medulla (Okada *et al.* 2001) and surrounds the fourth ventricle. The NTS is immunoreactive for c-fos after exposure to hypercapnia (Sato *et al.* 1992; Belegu *et al.* 1999; Berqin *et al.* 2000). Unilateral lesions of this region in humans causes reduced responses to hypercapnia (Morrel *et al.* 1999, 2001). Local perfusion of this region with AZ, causing pH changes, leads to increases in phrenic nerve discharge and increased blood pressure (Coates *et al.* 1993). The chemosensitivity of NTS neurons was only slightly reduced when hypercapnia was applied in the presence of a solution to block synaptic transmission (11.4 mM Mg^{2+}/0.2 mM Ca^{2+}) or electrical coupling (carbenoxolone 100 μM) (Nichols *et al.* 2008). However, as discussed earlier, these are weak criteria for establishing whether the NTS is a site of primary chemoreception.

The properties of, and coupling between, NTS neurons undergo maturation in early development (Vincent & Tell, 1997; Nichols *et al.* 2008). Thus, caution must be exercised when extrapolating functional roles of the NTS in chemosensitive reflexes at different stages of development. Nevertheless the NTS is a relay for peripheral chemosensory inputs (Nattie & Li, 2002) and is connected to almost all chemosensory and respiratory related nucleus. It is therefore positioned to integrate a number of different inputs and control both respiration and blood pressure during hypercapnia.

Paraventricular nucleus (PVN)

The hypothalamic paraventricular nucleus is located next to the third ventricle (Cham *et al.* 2005) and is the master controller of blood pressure regulation. Stimulation of the PVN excites the pressor areas of the rostral ventrolateral medulla (RVLM) (Coote *et al.* 1998), an important nucleus in blood pressure regulation during hypercapnia (Moreira *et al.* 2006). The PVN also projects to the preBötC, phrenic nucleus, and the spinal cord (Yeh *et al.*

1997; Mack *et al.* 2007), and acts upon these nuclei through the release of oxytocin (Mack *et al.* 2007). Neurons projecting to the phrenic nucleus express c-fos during hypercapnia (Berqin *et al.* 2000; Kc *et al.* 2002). PVN stimulation causes increased breathing frequency and amplitude and blood pressure (Yeh *et al.* 1997). Thus the PVN is poised to influence blood pressure and respiration through intermediate nuclei and motor neurons. At present, this evidence is at best suggestive and further evidence is required if it is to be considered a primary or even secondary site of chemoreception.

The preBötzinger Complex (preBötC)

The preBötC is the site of inspiratory rhythm generation (Smith *et al.* 1991) and may be intrinsically chemosensitive. In neonatal *in vitro* preparations all neuronal subtypes of the preBötC were affected by alterations in CO_2 and pH and though no discharge could be seen, they retained their membrane potential alterations in the presence of TTX, cadmium or calcium free solutions to stop synaptic activity (Kawai *et al.* 1996, 2006; Koizumi *et al.* 2010). In addition, focal acidosis of this region *in vivo* in adult animals also causes alterations in respiratory output (Solomon *et al.* 2000, 2003; Krause *et al.* 2009). However, as discussed above, this is only a weak criterion for establishing whether the preBötC is a primary chemosensitive area.

The retrotrapezoid nucleus (RTN)

RTN neurons are located at 130–230 μm from the ventral surface of the medulla and in the marginal glial layer (MGL) (Mulkey *et al.* 2004), with extensive secondary dendrites located within the MGL (Mulkey *et al.* 2004). They show c-fos immunoreactivity in response to elevated CO_2 (Berqin *et al.* 2000) and are exquisitely sensitive to CO_2 above 4% *in vivo* (Hewitt *et al.* 2004; Mulkey *et al.* 2004) and to hypercapnic acidosis *in vitro* (Mulkey *et al.* 2004). They are also activated by injections of AZ directly into this nucleus (to cause acidification) (Hewitt *et al.* 2004). The RTN shows connections to the preBötC (Mulkey *et al.* 2004) and either it or the closely localized pFRG acts as an expiratory oscillator (Janczewski & Feldman, 2006*a*,*b*; Pagliardini *et al.* 2011), and thus it is uniquely poised to alter respiration.

While RTN neurons remain responsive to CO_2 in the presence of a cocktail of antagonists (Mulkey *et al.* 2004), this is not conclusive evidence for their being intrinsically chemosensitive. CO_2-dependent release of ATP occurs in the vicinity of the RTN neurons. Unlike early reports that used Hepes buffered saline (Mulkey *et al.* 2006), recent evidence suggest that ATP plays at least a partial role in the chemosensitive responses of these neurons at ages P7–12 (Wenker *et al.*, 2010) and, in the adult, responses of RTN neurons to acidosis appear almost entirely dependent on the prior release of ATP (Gourine *et al.*, 2010). Thus their intrinsic chemosensitivity appears to alter with age. Though the primacy of the RTN is in question, it remains important as an integrator or relay, as loss of this nucleus severely blunts the response to inspired CO_2 (Marina *et al.* 2010). Whether the RTN is a primary chemosensory nucleus will remain unclear until identification and genetic manipulation of the chemosensory transducer has been achieved.

Introduction of human mutations associated with congenital central hypoventilation syndrome (CCHS) into the mouse *Phox2b* gene also suggests that the RTN has an important role in chemosensitivity (Dubreuil *et al.* 2009) as neonatal mice hetero- or homozygous for these mutations completely lack CO_2 chemosensitivity (Dauger *et al.* 2003; Ramanantsoa *et al.* 2011). However, the importance of the RTN for adult chemosensitivity is less clear as adult *Phox2b* heterozygotes have an intact response by P10 (Dauger *et al.* 2003) and homozygotes regain around 40% of the sensitivity to CO_2 (Ramanantsoa *et al.* 2011). In addition, c-fos studies show that the RTN is significantly more important in neonatal life than in adults (Belegu *et al.* 1999). Thus while the RTN may have an important primary role in neonatal chemosensitivity, it may switch to be more of an integrator of other chemosensory inputs in the adult. It must be noted that *Phox2b* mutations have far reaching effects in humans, affecting the entire peripheral chemosensory arc and other chemosensitive nuclei, and so caution is required when relating this mutation to a specific chemosensory nucleus unless it is targetted to a specific neuronal subpopulation (e.g. Ramanantsoa *et al.* 2011).

The marginal glial layer (MGL)

The primary cell type at the ventrolateral medulla surface is astrocytes. Presumed glial cells within the MGL depolarise in response to hypercapnia (Fukuda *et al.* 1978; Ritucci *et al.* 2005) and perfusion of the rostral VLM with fluorocitrate, a glial toxin, reduced the hypercapnic ventilatory response in anaesthetised rats (Erlichman *et al.* 1998). Injections of fluorocitrate into the RTN of conscious adult rats increased ventilation and heightened the response to CO_2, due to increased extracellular acidification (Holleran *et al.* 2001). However, 50% of the animals went into respiratory distress (Holleran *et al.* 2001) and for obvious reasons these experiments were discontinued. The results of this investigation could therefore have been biased towards animals where gliotransmitter dysfunction was not large enough to have an effect. Intraperitoneal injections of methoxysulphate, another glial toxin, into neonatal rats, which should affect a much larger proportion of glia compared to site directed injections, resulted in reduced levels of basal ventilation and their CO_2 response (Young *et al.* 2005).

Evidence is beginning to unveil the crucial role for glia in chemosensation (Gourine *et al.* 2010; Huckstepp *et al.* 2010*b*; Wenker *et al.* 2010). RTN astrocytes are intrinsically pH sensitive in neonates (Wenker *et al.* 2010) and in the adult (Gourine *et al.* 2010). Sub-pial astrocytes in the adult are CO_2 sensitive via Cx26 (Huckstepp *et al.* 2010*b*) and pH sensitive (via an unknown molecule). Blockade of ATP release (including from astrocytes) during hypercapnia has been shown to reduce the ventilatory response (Gourine *et al.* 2005, 2010; Huckstepp *et al.* 2010*a*,*b*; Wenker *et al.* 2010) and applications of exogenous ATP replicate the response to increased CO_2 (Gourine *et al.* 2005, 2010). Genetic evidence via the removal of the chemosensory transducers to demonstrate alterations in chemosensory reflexes in awake behaving animals is still required for full evaluation of these mechanisms. Nonetheless, neurons of all chemosensitive sites project dendrites to the MGL and many chemosensory nuclei express P2X and P2Y or are affected by ATP (Nieber *et al.* 1997; Sansum *et al.* 1998; Ralevic *et al.* 1999; Yao *et al.* 2000; Thomas *et al.* 2001; Whitlock *et al.* 2001; Fong *et al.* 2002; Lorier *et al.* 2004; Sergeeva *et al.* 2006; Cao & Song, 2007; Lorier *et al.* 2007). Thus, the MGL is poised to alter every aspect of chemosensory function and has recently been hypothesized to be a unifying factor at all chemosensory sites (Huckstepp *et al.* 2010*b*).

Leptomeninges

The leptomeninges, and in particular the pia mater, express Cx26 (Filippov *et al.* 2003; Huckstepp *et al.* 2010*b*) and are responsive to CO_2 (Huckstepp *et al.* 2010*b*), and thus they could contribute to chemosensory reflexes. The opening of Cx26 by CO_2 could allow sufficient release of ATP to diffuse into the parenchyma and activate receptors on superficial marginal glial cells and the dendrites of neurons. There is no evidence to support this possibility, but specific deletion of Cx26 within the leptomeninges may provide an answer as to whether this outer covering of the brain, rich in blood vessels, could be part of the chemosensory machinery.

The complexity of chemosensitivity

Table 2 summarizes and evaluates the contribution of the different brain areas to chemosensitive reflexes. It becomes readily apparent that each chemosensory reflex is subserved by at least two putative chemosensitive areas. What is the reason for such complexity and apparent redundancy? Developing the thinking of Nattie and colleagues (Nattie, 1999, 2000), we believe there are three possible explanations, as outlined below.

Complexity is only apparent owing to incomplete knowledge. Some of the putative and provisional chemosensitive areas may fail to make the grade when stricter definitions, developed in this review for chemosensory transducers and intrinsic chemosensory cells, are rigorously applied. It may therefore be that for some reflexes only a single primary chemosensitive nucleus is involved. Nevertheless, this seems unlikely and especially so for breathing where six different primary sensor nuclei have been proposed and three of these have either likely or provisional status.

Redundancy hypothesis. Nattie (2000) has proposed that the robust nature of the overall response to CO_2 depends on additive or greater interactions of inputs from multiple locations. The sensitivity of neuronal processing of hypercapnia may be enhanced if all of the neurons in the pathway were chemosensitive (Su *et al.* 2007). In addition, a multiplicity of chemosensory sites would provide greater overall stability (Li & Nattie, 2008*a*) by allowing for corrections of regional imbalances of blood flow and metabolism.

Multiple salient signals hypothesis – implications for parallel processing. A more attractive possibility is that the *apparent* redundancy enables more abstraction of information about physiological state. As the visual system abstracts and computes multiple aspects of the visual stimulus in parallel, the chemosensory system may do the same through parallel processing of multiple salient signals (Fig. 1). In addition to processing pH_i, pH_e, $[HCO_3^-]$, and P_{CO_2} separately, different chemosensors exhibit a range of response characteristics in both the temporal and concentration domains. Some chemosensors give sustained responses (Huckstepp *et al.* 2010*b*), whereas others respond in a transient fashion (Gourine *et al.* 2010). In breathing, for example, Cx26 in glia can report both increases and decreases of P_{CO_2} (Huckstepp *et al.* 2010*a*), whereas the RTN is only sensitive to CO_2 above 4% (Mulkey *et al.* 2004). Thus, different chemosensory transducers and nuclei have different 'set points' allowing them to respond to different magnitudes and absolute ranges of change in the relevant salient stimulus. This in turn may mean that tonic baseline input is required from at least some chemoreceptors to allow full expression of the systemic response (Nattie, 2000). Hypoxia can cause intracellular acidification of a range of neurons in the NTS and ventrolateral medulla. In the absence of peripheral chemosensors, this intracellular acidification is not sufficient to cause an enhancement of breathing (Putnam *et al.* 2004). The reasons for this are not fully understood, but this observation is consistent with the concept that a range of salient chemosensory signals can be processed in parallel to allow accurate computation of the physiological state.

We are a long way from having sufficient knowledge to understand at a systems level how the multiplicity of different transducing molecules in different areas would

Table 2. Summary of the chemosensitive physiological reflexes, the areas involved in mediating the chemosensitivity and the status of the evidence

Physiological/ behavioural function	Areas	Signalling molecules	Salient signals	Status of evidence as primary area
Breathing	*Retrotrapeziod nucleus*	Glutamate	pH_e	Provisional (neonatal) Secondary (adult)
	PreBötzinger Complex	Glutamate	pH	Putative
	Sub-pial astrocytes in the VLM	ATP	CO_2	Likely
			pH	Provisional
	Locus coeruleus	Noradrenaline	pH	Provisional
	Nucleus tractus solitarus	Glutamate	pH	Putative/ secondary
	Medullary raphé	Serotonin	pH	Contested
Cardiovascular	*Nucleus tractus solitarus*	Glutamate		Putative
	Paraventricular nucleus	Glutamate		Putative
Arousal/vigilance	*Tuberomammillary nucleus*	Histamine		Putative
	Lateral Hypothalamus	Orexin	pH_e	Putative
	Dorsal raphé	Serotonin	pH	Provisional
Fear	*Amygdala*		pH_e	Likely
	Locus coeruleus	Noradrenaline	pH_e	Putative

VLM, ventrolateral medulla; pH_e, extracellular pH; pH_i intracellular pH; unlabelled pH, unknown. Strength of evidence: likely > provisional > putative > contested.

allow the brain to compute the exact condition of the entire system. This is particularly relevant as metabolic and respiratory acidosis reconfigures the respiratory network in neonates in different ways (Okada *et al.* 2007). During metabolic acidosis, areas sensitive to changes in either pH_i or pH_e would be stimulated, whereas those areas sensitive only to HCO_3^- and CO_2 fluctuations would not. By contrast during respiratory acidosis all areas would be excited. During exercise, the reduction of pH arising from increases in anaerobic respiration are likely to be accompanied by a reduction in $[HCO_3^-]$ as it buffers lactate (Beaver *et al.* 1986). Thus only pH-sensitive sites would stimulate respiration whereas HCO_3^--sensitive sites might do the opposite. This general concept that chemosensory pathways are organized to process different types of salient information in parallel pathways has the capacity to allow greater adaptation of the system. By providing a system that monitors multiple facets of the same chemical reaction, the system may still adapt to changes when one of the signalling molecules of this reflex is already saturated.

Concluding remarks

In light of recent evidence we believe that chemosensation is at an exciting stage of development. To progress further we believe that the new criteria proposed here for establishing a chemosensory transducer and a primary chemosensory nucleus need to be rigorously applied across the whole field. We accept that this is potentially very difficult, especially in those cases where the transducer has not yet been identified. This rigorous approach will require targeted and inducible genetic deletions of the relevant molecules. Implicit in this approach is a re-evaluation of the salient chemosensory signals with the possibility that hitherto marginalized molecules (CO_2 and HCO_3^-) may ultimately play a larger role than suspected and that some putative chemosensors and chemosensory areas will be eliminated from the field.

In addition, careful quantitative characterization of the properties of the transducer molecules, along with the dynamic and quantitative outputs from the different nuclei involved, is needed. This level of detailed information is necessary to allow us to understand how different aspects of the chemosensory stimulus are detected and how the different aspects of information are processed. This will lay the groundwork for a future systems level understanding of chemosensation.

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