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Chemoreception and asphyxia-induced arousal

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Abstract

Arousal protects against the adverse and potentially fatal effects of asphyxia during sleep. Asphyxia stimulates the carotid bodies and central chemoreceptors but the sequence of events leading to arousal is uncertain. In this review, the theoretical mechanisms leading to arousal from sleep are briefly summarized and the issue of whether central respiratory chemoreceptors (CRCs) or other types of CO₂-responsive CNS neurons contribute to asphyxia-induced arousal is discussed. We focus on the role of the retrotrapezoid nucleus, the raphe and the locus coeruleus and emphasize the anatomical and neurophysiological evidence which suggests that these putative central chemoreceptors could contribute to arousal independently of their effects on breathing. Finally, we describe recent attempts to test the contribution of specific brainstem pathways to asphyxia-induced arousal using optogenetic and other tools and the possible contribution of a group of hypoxia-sensitive brainstem neurons (the C1 cells) to breathing and arousal.

Keywords

retrotrapezoid nucleus; raphe; locus coeruleus; optogenetics; sleep apnea

1. Introduction

Arousal protects against the adverse and potentially fatal effects of asphyxia during sleep (Dempsey et al., 2010). Selective activation of the carotid bodies (hypoxia) or preferential activation of central chemoreceptors (hypercapnia) is sufficient to cause arousal, including in man (Phillipson et al., 1977; Phillipson et al., 1978; Gleeson and Zvillich, 1992). Based on experiments in man, arousal from sleep in response to a variety of respiratory stimuli (hypercapnia, hypoxemia, resistive loading) is best predicted by the level of peak intrathoracic pressure during breathing efforts rather than by arterial chemistry (SaO₂ and CO₂) (Gleeson et al., 1990). Similarly, arousal from sleep occurs at about the same level of ventilation regardless of whether breathing is activated by carotid body stimulation with adenosine or by central chemoreceptor stimulation with hyperoxic hypercapnia (Gleeson and Zvillich, 1992). These human experiments are interpreted as evidence that arousal from sleep is the “consequence of increased ventilation” rather than a direct effect of the specific ventilatory stimulus (Gleeson and Zvillich, 1992). However, there are many consequences of ventilation and many ways in which brain activity could correlate with a given level of

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ventilation. The problem is to distinguish causality from correlation and to identify the neuronal circuitry responsible for the arousal.

In theory, each of the mechanisms illustrated in Figure 1 could contribute to asphyxia-induced arousal. The carotid bodies (CBs) and central respiratory chemoreceptors (CRCs) could cause arousal strictly via their activation of the brainstem respiratory pattern generator (RPG) and downstream connections between the RPG and wake-promoting pathways (concept of corollary discharge). There could also be connections between CB afferents or CRCs and wake-promoting pathways that by-pass the RPG. Other possibilities include the existence of pH/CO₂-sensitive neurons (central chemoreceptors, CCs) that regulate vigilance but are not directly involved in the regulation of breathing. Finally, sensory feed-backs from lung, chest and airways receptors could also contribute to arousal. Corollary discharge, for example, could explain observations that respiratory efforts, which reflect RPG output, predict the point of arousal during hypercapnia, hypoxia, selective carotid body stimulation and resistive loading (Gleeson et al., 1990; Gleeson and Zwillich, 1992). The concept of corollary discharge derives its earliest experimental support from the identification of neurons within the pons and forebrain that display respiratory modulated firing pattern independent of afferent feedback (Chen et al., 1991; Chen et al., 1992).

2. Defining central respiratory chemoreceptors (CRCs)

2.1. What are CRCs?

Before considering how central chemoreceptors might contribute to arousal, a brief update on the biology of these receptors is in order. In this review the term central chemoreceptors is used to describe any brain cell that responds to CO₂ or acidification. By contrast the term central respiratory chemoreceptors (CRCs) is used in reference to CNS cells that detect elevated CO₂ (directly or in a paracrine fashion) and stimulate breathing. The distinction is essential since we do not know yet if and to what extent the arousal effect of hypercapnia is mediated by CRCs.

Currently, neurons in the retrotrapezoid nucleus, medullary raphe, nucleus of the solitary tract (NTS) and locus coeruleus, as well as certain astrocytes, are examples of CRCs with the most evidential support (Nichols et al., 2008; Gourine et al., 2010; Guyenet et al., 2010; Gargaglioni et al., 2010; Hodges and Richerson, 2010a; Huda et al., 2012). According to the time-honored “reaction theory”, CO₂ works by proxy of pH i.e. via “proton receptors”, however, several lines of evidence suggest alternative mechanisms, such as receptors that detect molecular CO₂ and a bicarbonate-sensitive adenylate cyclase (Loeschcke, 1982; Guyenet et al., 2010; Huckstepp et al., 2010; Choi et al., 2012). Candidate proton receptors (i.e. connexins, ion channels such as ASIC channels, two-pore domain potassium channels or inwardly-rectifying potassium channels Kir4.1-Kir5.1, TRP channels, calcium channels) are widely expressed in brain (Askwith et al., 2004; Huckstepp et al., 2010; Wenker et al., 2010), and thus provide little indication of the location of the relevant CRCs (Guyenet et al., 2010). Moreover, CO₂ and/or protons directly depolarize astrocytes, particularly in the brainstem causing them to release transmitters such as ATP and polyphosphates (Holmstrom et al., 2013; Kasymov et al., 2013) that contribute to the activation of at least some of the CRCs (Gourine et al., 2010; Wenker et al., 2010). In addition, astrocytes regulate capillary flow in the brain (Peppiatt et al., 2006; Iadecola and Nedergaard, 2007) and the cerebral vasculature (arteriolar smooth muscle) dilates under the direct influence of acidification (Tian et al., 1995). These direct and indirect effects of CO₂ on the brain microvasculature (via glia and pericytes) have considerable potential to alter the response of CNS neurons to changes in PaCO₂.

2.2. Example of putative CRC: the retrotrapezoid nucleus

Whether protons or CO₂ are detected by neurons directly or via paracrine mechanisms, the key distinction between a CRC and generic pH-modulated CNS neurons is connectivity i.e. whether and to what extent these CO₂-sensitive neurons can activate the respiratory centers. The case of the retrotrapezoid nucleus is an apt illustration (Figure 2). These glutamatergic neurons have been defined by a combination of anatomical and electrophysiological criteria (Guyenet et al., 2010). The response of RTN neurons to CO₂ in vivo (0-10 Hz in anesthetized rats (Guyenet et al., 2005)) is larger than that of other candidate central chemoreceptors recorded under similar conditions but otherwise unremarkable (Elam et al., 1981; Depuy et al., 2011). A more meaningful attribute of RTN neurons is that this dynamic range is achieved over a mere 0.2 units of arterial pH in vivo resulting in a discharge rate change of ~0.5 Hz per 0.01 pH (Guyenet et al., 2005). However, the most important characteristics of RTN neurons are a) their glutamatergic nature, b) their heavy and probably monosynaptic input to the respiratory rhythm and pattern generator, c) the fact that selective activation of these neurons produces large increases in breathing in intact rodents suggesting a very high synaptic gain and, d) the fact that selective inhibition of these neurons greatly attenuates the hypercapnic ventilatory reflex without changing baseline breathing (Mulkey et al., 2004; Abbott et al., 2009; Marina et al., 2010; Abbott et al., 2011; Bochorishvili et al., 2012). Last but not least, when the development of RTN neurons is prematurely aborted by genetic means in mice, the hypercapnic ventilatory response is eliminated at birth and permanently reduced by about 65% thereafter (Ramanantsoa et al., 2011).

RTN neurons are intrinsically responsive to acid in neonate rodents as their response persists after blockade of calcium-dependent exocytosis or after complete isolation (Onimaru et al., 2012; Wang et al., 2013). This intrinsic property, demonstrated in rats and mice, seems to be present very early during development (Thoby-Brisson et al., 2009). A second factor believed to contribute to the CO₂ responsiveness of RTN neurons in vivo is a purinergic input from surrounding pH-sensitive glial cells (Mulkey and Wenker, 2011). This glial regulation seems weak in the neonate (~25% of total acid sensitivity (Mulkey and Wenker, 2011)). It may develop later which could perhaps explain the relative weakness of the hypercapnic ventilatory response during the post-natal period (Davis et al., 2006; Thoby-Brisson et al., 2009; Onimaru et al., 2012).

Although RTN neurons qualify as CRCs by virtue of the fact that they are activated by a rise in the PCO₂ within the surrounding neuropil, this property does not fully define their contribution to the regulation of breathing. These neurons are also a point of convergence for multiple synaptic inputs, most notably from the carotid bodies (Takakura et al., 2006). In addition, they receive feedback inhibition from lung stretch afferents and the RPG (Guyenet et al., 2005; Moreira et al., 2007) and they are activated by serotonergic neurons (Mulkey et al., 2007; Dias et al., 2008) and hypothalamic orexinergic neurons that may also be directly or indirectly activated by CO₂ (Williams et al., 2007; Dias et al., 2009; Sunanaga et al., 2009; Lazarenko et al., 2011). Of particular significance, RTN neurons are activated by mild, most likely isocapnic, exercise (Barna et al., 2012) and by posterior hypothalamic stimulation (Fortuna et al., 2009). These latter observations need to be expanded and the RTN connectome completed but they strongly suggest that RTN neurons are also regulated by CO₂-independent inputs. In other words, RTN neurons likely convey to the respiratory network a mixed input, part “chemical drive to breathe” (CO₂-dependent), part “waking drive to breathe” i.e. non-CO₂ regulated. Figure 2 summarizes these ideas. Other putative CRCs, to the extent that they are neurons rather than glia, are undoubtedly also subject to cell-specific inputs and regulations over and above their ability to respond to local changes in PCO₂. The locus coeruleus is a particularly well-documented example (e.g. (Aston-Jones and Cohen, 2005)).

2.3. Other CRCs

Topical acidification of numerous lower brainstem regions increases ventilation, which has been interpreted as evidence for the existence of multiple CRCs besides raphe or RTN neurons. This topic has been recently reviewed (Nattie, 2011) and will not be revisited here except to point out that the concept derives some additional support from recent evidence that acid- (or CO₂-) sensitive ATP-releasing brainstem astrocytes are distributed over a large portion of the ventral surface of the medulla oblongata (Gourine et al., 2010; Kasymov et al., 2013). This entire region, not just the RTN or the raphe, contains cells that express Fos in animals exposed to hypercapnia (Teppema et al., 1997; Okada et al., 2002). Many such cells are intimately associated with superficial blood vessels and have not been identified as either neurons or glia (Okada et al., 2002) but others could conceivably be neurons with CRC properties (da Silva et al., 2010) or could be “non-respiratory” chemoreceptors i.e. neurons that are directly or indirectly pH-activated and have functions other than to produce breathing stimulation. It should be noted however that these speculations are inconsistent with prior evidence that eliminating a small subset of *egr2*-derived neurons including the RTN eliminates the chemoreflex in mice at birth, the reason being that regions of the medulla oblongata caudal to RTN do not derive from rhombomeres 3 or 5 (Ramanantsoa et al., 2011). Brainstem serotonergic neurons and the locus coeruleus are presumed to function as CRCs (Gargaglioni et al., 2010; Hodges and Richerson, 2010b), their role in arousal caused by asphyxia will be discussed later.

3. Integration between peripheral and central chemoreceptor inputs

Peripheral and central chemoreceptors have hyper-additive ventilatory effects in intact, awake and resting goats or dogs (Forster et al., 2008; Blain et al., 2010). In man, a preponderance of evidence suggests that the interaction is merely additive (e.g. (Cui et al., 2012) and references herein). A more consistent finding across species including man is that carotid body denervation causes an immediate, large (~70%) and very long-lasting hypoventilation and reduction of the hypercapnic ventilatory response (Forster and Smith, 2010). The importance of the carotid bodies to the hypercapnic ventilatory response is also highlighted by experiments in dogs in which extracorporeal perfusion of an intact carotid body was used to acutely inhibit or stimulate the peripheral chemoreflex (Blain et al., 2010). In this paradigm, carotid body inhibition reduced the ventilatory response to CNS hypercapnia by 80% and carotid body stimulation increased this response by roughly two-fold.

In anesthetized mammals central and peripheral chemoreceptors typically have roughly additive effects on ventilation. A hypoadditive hypercapnic-hypoxic interaction on breathing has also been reported, first in a hypothermic arterially-perfused midcollicular transected rat preparation (Day and Wilson, 2009) and more recently in urethane-anesthetized atropinized rats (Tin et al., 2012).

In sum, in conscious resting mammals, the effects of moderate levels of central and peripheral chemoreceptor stimulation on breathing are at least additive. The nature of the interaction is species-specific and varies depending on factors such as anesthesia or the type of experimental preparation. The interaction between the effect of central and peripheral chemoreceptors on arousal is yet to be examined. The hyperadditivity of the ventilatory effects of central and peripheral chemoreceptors is not well-understood in neurophysiological terms. Potential explanations include the convergence of carotid body inputs on CRCs, a fact documented in the case of RTN neurons, the locus coeruleus or neurons located in the nucleus of the solitary tract. The surprisingly large contribution of the carotid bodies at rest could result from a threshold effect during central integration. More specifically, the resting level of CNS PCO₂ could be essential to depolarize CRC neurons

towards their discharge threshold, enabling even a small degree of additional excitatory input from the carotid bodies to produce a comparatively large increase in central respiratory drive relative to the resting state. This notion is supported by the results of recent experiments performed in the retrogradely perfused rat preparation in which strong stimulation of the carotid bodies could overcome the apneic effect of CNS hypocapnia (Fiamma et al., 2013). So far, hypoadditivity has been observed in anesthetized vagotomized or reduced and vagotomized preparations subjected to high levels of stimulation of central chemoreceptors. This type of interaction has not been observed at rest in awake mammals but could conceivably exist under conditions such as intense exercise or very abnormal levels of blood gases.

4. Aversive quality of asphyxia in the awake state

Intero- and extero-nociceptive stimuli that are perceived as painful or aversive when awake typically produce arousal from sleep. Whether hypercapnia and hypoxia are aversive stimuli in awake mammals is therefore relevant to the ability of these stimuli to cause arousal from sleep, irrespective of their specific effects on breathing. In this section we briefly review the evidence which indicates that acute stimulation of peripheral and or central chemoreceptors is aversive in the awake state and that the arousal-promoting effects of these stimuli can be dissociated to some degree from their effects on the mechanics of breathing.

4.1. Hypercapnia and the “urge to breathe”

Mechanically ventilated quadriplegic humans exhibit sleep-to-wake responses to CO₂ (Ayas et al., 2000). In awake humans, periods of air starvation (e.g. diving, breath-holding), inadequate alveolar ventilation (e.g. COPD) and experimentally induced hypercapnia evokes feelings of ‘air hunger’ (i.e. the conscious perception of the urge to breathe) (Parshall et al., 2012). Hypercapnic stimulation in paralyzed mechanically ventilated individuals produces intolerable air hunger when end-expiratory PCO₂ reaches a modest 40-46 mmHg (Banzett et al., 1990; Gandevia et al., 1993). In these experiments the perception of the elevated CO₂ occurred after a sufficiently long delay to suggest that the increase in CNS pCO₂, therefore some form of central chemoreceptors albeit not necessarily “respiratory”, was of critical importance. Together these studies demonstrate that the pulmonary and extra-pulmonary feedbacks related to increased ventilation are not required for hypoxia and hypercapnia to produce highly aversive effects in awake man.

4.2. Forebrain vs. brainstem chemoreceptors and CO₂-induced arousal

Acid-sensitive channels are widely expressed in brain (Holzer, 2009). In mice, severe hypercapnia (10% inspired CO₂) produces behavioral indices of fear (Ziemann et al., 2009) which are greatly attenuated in *ASIC1a*^{-/-} mice and can be restored by reintroducing ASIC1a channels specifically in the amygdala of the mutant strain. Interestingly, the hypercapnic ventilatory response of *ASIC1a*^{-/-} mice was unchanged, suggesting that the aversive quality of the stimulus was not primarily elicited by the activation of CRCs or by afferent feedback from the chest and lungs. These results and the human studies described earlier indicate that hypercapnia is perceived as aversive and that the sensation, even in rodents, is not necessarily linked to the degree of increase in lung ventilation. Furthermore, Ziemann et al. suggests that the amygdala is a chemosensor that detects carbon dioxide and acidosis via ASIC1a channels to elicit fear behavior. Is a forebrain mechanism responsible for the aversive quality of acute hypercapnia and CO₂-induced arousal in man? The case of congenital central hypoventilation syndrome (CCHS) and recent evidence that patients with bilateral amygdala damage experience air hunger and panic attacks when exposed to CO₂ (Feinstein et al., 2013) would seem to argue against this possibility.

CCHS is typically caused by mutations of homeodomain transcription factor Phox2b, most commonly an expansion of the poly-alanine tract (Amiel et al., 2003; Weese-Mayer et al., 2010; Goridis and Brunet, 2010). Affected individuals exhibit autonomic dysregulation, and experience partial to complete loss of both hypercapnic and hypoxic ventilatory reflexes, abnormal fluctuations of arterial PCO₂ and, in the most serious cases (e.g. 27 alanine repeats), potentially fatal respiratory failure while asleep. Importantly, CCHS patients do not experience air hunger during CO₂ exposure and breath-holding (Shea et al., 1993), but they do report breathlessness during exercise (Shea et al., 1993; Spengler et al., 1998).

The expression of Phox2b is required for the differentiation of peripheral visceral afferent pathways, including the carotid bodies and their afferent innervation. Phox2b expression is also required for the correct development of the nucleus of the solitary tract, all lower brainstem catecholaminergic neurons and a variety of pontine regions implicated in autonomic responses and the control of facial muscles (Brunet and Pattyn, 2002; Goridis and Brunet, 2010). The RTN is especially vulnerable to Phox2b polyalanine expansion in mice; selective expression of Phox2b-27ala by egr-2 domain progenitor cells prevents RTN development and produces animals that survive but lack a hypercapnic ventilatory response at birth and for several weeks thereafter (Ramanantsoa et al., 2011). The viability of mice lacking RTN neurons, despite the virtual absence of the central hypercapnic ventilatory response, suggests that CCHS in humans could involve other deficits, which is not surprising given that virtually every neuron involved in carotid body chemoreflexes express Phox2b (type I glomus cells, sinus nerve afferents, NTS relay neurons, RTN and C1 neurons) and presumably depend to some extent on this transcription factor for their proper development. Nonetheless, the CCHS is instructive with regard to how central chemoreceptor stimulation might produce arousal from sleep or air hunger. As Phox2b is not implicated in the development of the brain above the pons, it is unlikely that hypercapnia-induced arousal or the sensation of air hunger is primarily initiated by direct acidification of neurons located rostral to the pons (i.e. cortex, hippocampus, amygdala or hypothalamus (including the orexin neurons)). In fact, CCHS suggests that both CO₂-induced arousal and air hunger in man requires the activation of neurons located within the lower brainstem but no higher in the neuraxis. Consequently, these effects may have little in common with the above discussed ASIC1a-mediated fear responses evoked by hypercapnia in mice.

4.3. The carotid bodies and arousal

Hypoxia and airway occlusion in sleeping dogs causes arousal (Bowes et al., 1981a; Bowes et al., 1981b). This effect is greatly attenuated after removal of the carotid bodies, showing that these organs contribute to hypoxia-induced arousal. Mild hypoxia produces air hunger comparable to hypercapnia in man under conditions when the effect of hypoxia is not mitigated by concurrent hypocapnia (Moosavi et al., 2003). The perception of mild hypoxia diminishes with prolonged exposure (5-15 mins, PetO₂ ~ 50mmHg), mirroring the bi-phasic respiratory response to hypoxia (Moosavi et al., 2004). Considering that carotid body discharge is constant throughout similar periods of hypoxia, at least in anesthetized cats (Kou et al., 1991), the gradually declining sensation of hypoxia suggests that changes in the central processing of peripheral chemoreceptor drive during hypoxia, possibly via general CNS depression, attenuates the arousal and respiratory effects of the stimulus. In other mammals, acute selective stimulation of carotid chemoreceptors produces the defense response in conscious and lightly anesthetized rats and cats (Hilton and Marshall, 1982; Marshall, 1987; Marshall, 1994; Marshall, 1999), at even modest levels of hypoxemia (PaO₂ of 50-60 mmHg in rats). This response is not limited to respiratory stimulation and includes autonomic effects such as increased cardio-vagal and cardio-sympathetic activity, regional vasoconstriction or vasodilatation, vocalizations, piloerection and escape behavior that are indicative of the noxious quality of the stimulus. Aspects of the defense response are also

elicited in conscious mammals by noxious environmental stimuli and by stimuli that are emotionally stressful (Marshall, 1999), which supports potential parallels in the central processing of pain and asphyxia that are likely to be independent of respiratory effects.

4.4. Pulmonary and extra-pulmonary feedback

The aversive content of hypercapnia can be increased or decreased by changing pulmonary feedback independent of ventilation (reviewed in (Widdicombe, 2009)). This, in addition to the body of literature discussed above, has led to the hypothesis that the perception of air hunger is a manifestation of the mismatch between central chemoreceptor/respiratory drives and the sensation of ventilation (Lansing et al., 2009). If this is correct, where in the neuraxis does this integration occur?

Air hunger, and other aspects of dyspnea, is associated with activation of various aspects of the limbic and paralimbic system of the brain (Liotti et al., 2001; Brannan et al., 2001), however interconnections within the pons and medulla are a more probable site for integration of afferent feedback with central chemoreceptor/respiratory drive, such the parabrachial complex. Neurons in the PB complex express Fos following hypercapnia and hypoxia (Teppema et al., 1997; Berquin et al., 2000) and lesions of the PB complex in rats reduces the ventilatory response to hypoxia and hypercapnia without changing resting ventilation (Mizusawa et al., 1995). Neurons in the ventrolateral PB/Kölliker-Fuse subnuclei project to the RPG and respiratory motor neurons (Yokota et al., 2007) and chemical stimulation of these subnuclei produce various respiratory responses in anesthetized rats (Chamberlin and Saper, 1994), underscoring the role of this region in respiratory regulation (Chamberlin, 2004). Some PB subnuclei (medial PB/pre-coeruleus region) are indispensable for arousal, specifically the maintenance of waking states through projections to the basal forebrain (Fuller et al., 2011). The lateral PB complex which consists of several subregions plus the Kölliker-Fuse nucleus, see (Fulwiler and Saper, 1984)) integrates a polysynaptic input from the carotid body (Song et al., 2011), input from visceral afferents activated by increased ventilation by way of the NTS and the lamina 1 neurons of the spinal cord dorsal horn (Craig, 1995; Kubin et al., 2006). The lateral PB complex also receives direct inputs from the RPG (Chamberlin and Saper, 1998) and CRCs (e.g. RTN and serotonergic neurons) (Steinbusch, 1981; Miller et al., 2011; Bochorishvili et al., 2012), as well as a variety of inputs from the forebrain (Moga et al., 1990). In turn, the lateral PB innervates the forebrain, including the basal forebrain and targets the lateral hypothalamic area, amygdala and periaqueductal grey matter (Fulwiler and Saper, 1984). These efferent connections could potentially serve as a relay between chemoreceptors (peripheral and central), central respiratory drive, cardiopulmonary afferents, and forebrain structures that regulate arousal. Figure 3 illustrates the idea that the PB complex integrates central chemoreceptor drive (from RTN neurons for example), the ongoing activity of the RPG, and feed-back from pulmonary and extrapulmonary receptors and communicates disparities between central drive and peripheral feedback to higher brain regions, some of which were highlighted earlier. Consistent with this function, glutamatergic neurons in the LPB have recently been shown to be crucial for the arousal from sleep in response to hypercapnia (Kaur et al., 2012; Kaur et al., 2013); similar mechanisms may also important in the perception of air hunger.

5. C1 neurons, central oxygen sensing and responses to hypoxia

Based on the expression of Fos (Larnicol et al., 1994; Teppema et al., 1997; Berquin et al., 2000), hypoxia could cause arousal through a number of interconnected pathways. Carotid body afferents primarily target the commissural NTS, but also have sparse projections throughout the ventrolateral medulla (Finley and Katz, 1992). C1 neurons are a functionally heterogeneous collection of adrenergic neurons in the ventrolateral medulla that, collectively, mediate homeostatic autonomic responses to hypotension, hypoglycemia,

infection and probably pain (Guyenet, 2006). Though these neurons express enzymes needed to produce catecholamines, acutely stimulated C1 neurons release glutamate (Depuy et al., 2013). Many C1 neurons are robustly activated by carotid body stimulation and hypoxia as demonstrated by single-unit recordings (Koshiya et al., 1993; Reis et al., 1994; Sun, 1996) and Fos expression studies (Erickson and Millhorn, 1994; Sun, 1995; Hirooka et al., 1997). The C1 region is innervated by neurons in the commissural NTS and may be directly innervated by carotid body afferents (Finley and Katz, 1992; Aicher et al., 1996). C1 neuron activation makes an important contribution to the activation of sympathetic nerves during carotid body stimulation (Schreihöfer and Guyenet, 2000). C1 neurons are directly activated by brainstem hypoxia and their activation presumably contributes to the Cushing response (Dampney et al., 1979; Dampney and Moon, 1980; Guyenet and Brown, 1986; Sun and Reis, 1994).

In addition to its effect on peripheral sympathetic nerve activity, C1 neuron stimulation increases breathing frequency substantially (Abbott et al., 2013b) (Figure 4). The respiratory stimulation caused by C1 neuron activation was occluded when mice were exposed to hypoxia (10% O₂) but not hypercapnia (6% CO₂), despite significantly higher basal ventilation under hypercapnia. According to this evidence, the C1 neurons that activate breathing are preferentially activated by hypoxia vs. hypercapnia, and may contribute to the ventilatory response induced by hypoxia, hypotension and pain (Abbott et al., 2013b).

As well as a putative contribution to the hypoxic ventilatory response, C1 neurons likely contribute to many additional responses induced by hypoxia including epinephrine and corticosterone release and, probably, brown fat down-regulation, in part through projections to brain regions such as the paraventricular nucleus of the hypothalamus, the intermediolateral cell column and the dorsal motor nucleus of the vagus (Morrison et al., 1991; Sun and Reis, 1994; Li et al., 1996; Verberne and Sartor, 2010; Ritter et al., 2011; Madden et al., 2013; Abbott et al., 2013b). C1 neurons also innervate the locus coeruleus, the ventrolateral periaqueductal gray matter, the lateral PB, and the dorsomedial nucleus of the hypothalamus and perifornical region, which contains the orexinergic neurons (Card et al., 2006; Abbott et al., 2013b). These brain regions form an interconnected network of neurons that receive and disseminate neural signals influencing, among other things, arousal and sleep-wake patterns, and the autonomic, respiratory and behavioral components of the defense response (Carrive, 1993; Saper, 2002; Sakurai et al., 2010; Kuwaki and Zhang, 2010; Nattie and Li, 2010; Fuller et al., 2011; Berridge et al., 2012; Dampney et al., 2013). Generally, this network is likely to contribute to both state-dependent changes in breathing and the hypercapnic and hypoxic ventilatory responses (Nattie and Li, 2010) and the arousal-promoting effects of chemoreceptor activation.

6. C1 and RTN neurons, and asphyxia-induced arousal

We used optogenetic techniques (Yizhar et al., 2011) to test the possibility that activation of C1 and/or RTN neurons could produce arousal in rats (Abbott et al., 2013a). In these experiments RTN and C1 neurons were selectively activated using the light sensitive cation channel channelrhodopsin-2 while recording blood pressure (by radio-telemetry), breathing (by unrestrained plethysmography) and sleep-wake patterns (by electroencephalogram and neck muscle electromyography) (Figure 5). Photostimulation during both rapid and non-rapid eye movement sleep (REM and NREM sleep) produced arousal (i.e. sleep-to-wake-transitions), along with the expected cardiorespiratory activation (Kanbar et al., 2010). Interestingly, photostimulation trials during REM sleep caused arousal, but with reduced reliability relative to NREM sleep (83% in NREM sleep vs. 42% in REM sleep), even though the cardiorespiratory effects of photostimulation were mostly absent in this sleep state. This observation suggests that arousal caused by photostimulation of C1 and RTN

neurons is not entirely dependent on cardiorespiratory stimulation (i.e. the activation of afferent feedback mechanisms). This interpretation is also supported by the observation that systemic delivery of an alpha-1 adrenergic antagonist significantly reduced the cardiorespiratory effect of stimulation, but had no effect on the reliability of arousal during photostimulation. This work has a number of limitations. Most significantly, 80% of the neurons expressing ChR2 were C1 cells with the balance being RTN neurons, consistent with the selectivity of the promoter used to drive ChR2 expression (Hwang et al., 2001; Abbott et al., 2009). This issue prevented us to ascertain the relative contribution of C1 vs. RTN neuron stimulation to arousal. However, given that C1 and RTN neurons are activated to various degrees both by hypoxia and hypercapnia, this study suggests that the activation of these neurons under these conditions contributes to arousal.

Generally speaking, RTN neurons innervate respiratory-related regions of the brainstem, with an apparent specificity not seen in other candidate respiratory chemoreceptors (Samuels and Szabadi, 2008; Depuy et al., 2011; Bochorishvili et al., 2012). However the ventral respiratory column of the medulla oblongata also contains polymodal neurons that may well be implicated in arousal (Langhorst et al., 1996). The possibility that such neurons are targeted by the RTN in addition to respiratory neurons has not been ruled out. C1 neurons target a variety of regions that regulate arousal, as already described. C1 and RTN neurons have largely distinct projections and target a few common areas. Of distinction, RTN and C1 neurons both innervate overlapping regions of the lateral PB (Abbott et al., 2009; Bochorishvili et al., 2012). As highlighted earlier, the lateral PB may integrate central chemoreceptor/respiratory drive, as well as input from hypoxia-activated C1 neurons and afferent feedback. This structure contributes to CO₂-induced arousal in mice (Kaur et al., 2012; Kaur et al., 2013) and thus is an attractive neural substrate for some aspects of the arousal caused by C1 and RTN stimulation.

We speculate that the activation of C1 neurons during asphyxia contributes to arousal, by activating the pathways illustrated in figure 6, which in turn facilitates a corrective ventilatory response through state-dependent synaptic inputs to the brainstem, such as the orexinergic system. A possible scenario for the role of RTN neurons in arousal is shown in figure 3.

7. The raphe, the locus coeruleus and asphyxia

Serotonin and noradrenaline have well established roles in the regulation of neuronal excitability throughout the CNS. The excitability of the lower brainstem respiratory network and respiratory motor neurons is prominently regulated by these neuromodulators but also by histamine, substance P and orexin (Doi and Ramirez, 2008). These neuromodulators are a likely cause of state-dependent variations in breathing (Horner, 2009).

Medullary serotonergic neurons innervate the RPG and respiratory motor neurons and these direct and powerful excitatory inputs are likely to account for the well-recognized importance of serotonergic neurons in all aspects of breathing including central respiratory chemoreception (Ptak et al., 2009; Hodges and Richerson, 2010a; Depuy et al., 2011; Ray et al., 2011). In vitro and in culture, most serotonergic neurons from rats and mice respond to physiological shifts in pH with a 0.5-2 Hz increase in firing (~ 300% of baseline activity) (Wang et al., 1998; Wang et al., 2001). Medullary serotonergic neurons are typically unresponsive to CO₂ in halothane-anesthetized rats or isoflurane-anesthetized mice (Mulkey et al., 2004; Depuy et al., 2011) but a small fraction of medullary raphe neurons are CO₂-activated in conscious drug-free cats (Veasey et al., 1995). These discrepancies may be partly the result of different experimental approaches or could be interpreted as evidence that CO₂ sensitivity in vivo is limited to a subset of serotonergic neurons whose precise

location is not yet established. Underscoring the importance of serotonergic neurons in the hypercapnic ventilatory response, genetic ablation of these neurons (*Lmx1b^{f/fp}* mice) reduces the hypercapnic ventilatory responses by 50% (Hodges et al., 2008), and pharmacogenetic inhibition of serotonergic neurons reduced the hypercapnic ventilatory response by ~50% (Ray et al., 2011).

Like serotonin, noradrenaline has a well-documented facilitatory effect on the respiratory network from the level of the rhythm generator to motor neurons (e.g. (Doi and Ramirez, 2010)). The CO₂ response of adult LC neurons is modest in vitro and under anesthesia (0.5-1 Hz increase for a 5% increase in CO₂, ~100% of baseline firing) (Elam et al., 1981; Pineda and Aghajanian, 1997; Gargaglioni et al., 2010), but hypercapnia in conscious rats does produce vigorous Fos expression in LC neurons (Teppema et al., 1997), and destruction of LC neurons using 6-hydroxydopamine reduces the hypercapnic ventilatory reflex (by 40%) without affecting the response to hypoxia (Biancardi et al., 2008; Biancardi et al., 2010). In fact, ablation of either serotonergic or LC neurons has no effect on the hypoxic ventilatory response despite the fact that some of these neurons express Fos following carotid body stimulation and therefore must be vigorously activated by this stimulus (Erickson and Millhorn, 1994).

The role of serotonin and locus coeruleus neurons in CO₂-induced arousal has been reviewed previously (Buchanan and Richerson, 2009). A recent study has shown that *Lmx1b^{f/fp}* mice, which lack serotonergic neurons, have delayed arousal (from NREM sleep) in response to normoxic hypercapnia. Importantly, these animals have normal arousal responses to hypoxia, sound, and air puff (Buchanan and Richerson, 2010), showing that the effect was not caused by a generalized deficiency in arousability *per se*. However, *Lmx1b^{f/fp}* mice have greatly reduced hypercapnic ventilatory responses and an intact response to hypoxia (Hodges et al., 2008), thus the selective deficit in the arousal response to hypercapnia could also be a consequence of reduced breathing efforts or reduced RPG activation.

9. Sudden infant death syndrome, chemoreceptors and arousal

SIDS is tentatively attributed to the rare and fateful combination of a genetic predisposition, an immature and inherently unstable respiratory network and environmental factors (triple risk model)(Becker, 1990; Kinney and Thach, 2009). A defect in asphyxia-induced arousal possibly contributes to a fraction of SIDS cases (for recent reviews:(Kinney and Thach, 2009; Duncan et al., 2010; Darnall, 2013; Porzionato et al., 2013)) although many other causes of death are also being considered (see above mentioned reviews). The failure to arouse may be partly related to a dysfunction of peripheral and /or central chemoreceptors. For example, hypoplasia of the carotid bodies has been observed in SIDS, particularly in the case of prematurely born infants who may have been exposed to intermittent hyperoxia (Gauda et al., 2007; Porzionato et al., 2013). CNS defects are also suspected to contribute to SIDS. The evidence is largely of a postmortem neurohistological nature and typically relies on few specimens. The most effort has been dedicated to the serotonergic system. Abnormally high number of neurons and a reduction of selected serotonergic receptor subtypes have been reported suggesting delayed or abnormal development of these neurons (Duncan et al., 2010). In neonate rats, the ability to resuscitate following repeated bouts of severe hypoxia is compromised by severe lesions of serotonergic neurons (Cummings et al., 2011). Since CNS serotonergic neurons are required for CO₂-induced arousal in mice (Buchanan and Richerson, 2010), the collective evidence suggests that a defect in the development of the serotonergic system in man could perhaps contribute to the failure to arouse or to resuscitate via gasping and therefore to SIDS. Other brain regions or

transmitters (e.g. cholinergic system, retrotrapezoid nucleus) have also been found occasionally abnormal in SIDS (Lavezzi et al., 2012).

10. Conclusion

Although chemoreceptor stimulation is the initial trigger of asphyxia-induced arousal, the CNS pathways involved in this life-saving reflex are not well understood. The difficulty stems in part from our still patchy understanding of the cellular basis of central chemoreception (respiratory and otherwise) and of the circuitry engaged by the carotid bodies past the first synapse made by the primary afferents within the nucleus of the solitary tract. The apparently straightforward CRC concept hides a highly complex network of several types of neurons that may have little in common besides the property of responding to changes in local PCO₂. Each of the putative CRC highlighted in this review innervates multiple components of the respiratory network as well as brain regions implicated in arousal. Consequently, locus coeruleus, serotonergic, C1, and RTN neurons could contribute to asphyxia-induced arousal via their effects on breathing as well as independently of their ability to drive respiratory activity. Recent loss or gain of function experiments performed on serotonergic neurons (Buchanan and Richerson, 2010) or C1/RTN neurons (Abbott et al., 2013b) illustrate the difficulty of interpreting the effects of perturbations to neurons that have the necessary connectivity to produce arousal via purely central mechanisms, but also directly contribute to asphyxia-induced breathing stimulation. A key challenge going forward will be to determine whether the effects of CRCs on vigilance can be dissociated from their effects on arousal. This appears to be the case because, according to a very recent study, lesions of the parabrachial nucleus in mice reduce the probability of arousal to hypercapnia without changing the ventilatory response to this stimulus (Kaur et al., 2013).

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Highlights

- Theories of how chemoreceptor stimulation might produce arousal are summarized.
- Properties of central chemoreceptors are evaluated.
- We focus on the retrotrapezoid nucleus, C1 neurons raphe, and locus coeruleus.
- Recent gain and/or loss of function experiments performed in rodents are evaluated.

Highlights

- The various theories regarding how chemoreceptor stimulation might produce arousal are briefly summarized.
- We provide a brief update on what distinguishes central respiratory chemoreceptors from other CO₂ sensitive cells or neurons using the retrotrapezoid nucleus as example.
- The contribution of the retrotrapezoid nucleus, the raphe and the locus coeruleus to respiratory chemoreflexes and CO₂-induced arousal is discussed in the light of recent optogenetic and other gain or loss of function experiments. We particularly emphasize anatomical and neurophysiological evidence which suggests that these putative central chemoreceptors could contribute to arousal independently of their effects on breathing.
- The possible contribution of a group of hypoxia-sensitive brainstem neurons (the C1 cells) to breathing and arousal is evoked.

Figure 1. Putative mechanisms contributing to asphyxia-induced arousal

The arrows represent mono- or poly-synaptic connections between the carotid bodies (CB), central respiratory chemoreceptors (CRCs) and pathways that regulate arousal and vigilance. Wake-promoting pathways could be neurons in the reticular formation of the medulla, hypothalamic vigilance-regulating neurons, neurons in the parabrachial region, the locus coeruleus, serotonergic neurons, or all of the above. Central chemoreceptors (CC) refer to CO₂-modulated neurons that might mediate non-respiratory responses to hypercapnia such as arousal, fear or panic. Activation of the respiratory pattern generator (RPG) may drive arousal pathways directly (efferent copy theory) or produce arousal via sensory afferent feedback mechanisms engaged by the rise in lung ventilation. Mechanisms activating wake-promoting pathways are unlikely to be mutually exclusive.

Figure 2. An example of central respiratory chemoreceptor: the retrotrapezoid nucleus

Schematic representation of the known inputs and properties of retrotrapezoid neurons. Their sensitivity to CO_2 is attributed to at least four components, an intrinsic sensitivity to acid (1), a paracrine sensitivity to CO_2 mediated by the surrounding glia (2), excitatory inputs from CNS neurons that are directly or indirectly activated by CO_2 (3) and excitatory inputs from the carotid bodies (4). CO_2 may also have direct effects on the local microcirculation (not represented) that may influence the response of these neurons to changes in PaCO_2 . The schematic also indicates that retrotrapezoid (RTN) neuron activity is increased during exercise (Barna et al., 2012) and subject to feedback inhibition from the RPG and lung stretch afferents (for details see (Guyenet et al., 2010) and references therein).

Figure 3. Hypothetical contribution of the RTN to air hunger and asphyxia-induced arousal from sleep

RTN neurons respond to hypercapnia and hypoxia via a direct effect of CNS PCO_2 and via inputs from the carotid bodies. RTN neuron activation increases breathing via direct projections to the RPG, which includes neurons in the ventrolateral medulla (VLM) and pons (KF-Kölliker fuse and IPB-lateral parabrachial). Elevated ventilation caused by RTN stimulation increases afferent feedback to the nucleus of the solitary tract (NTS) and IPB, where the information is integrated with central chemoreceptor/respiratory drive from the RTN and other chemoreceptors (not shown) and possibly forwarded to regions of the brain regulating arousal and perception of air hunger. Such regions probably include the periaqueductal grey, amygdala and hypothalamus. Additional projections from the RTN to polymodal neurons located in the medullary reticular formation could also contribute to arousal (not shown).

Figure 4. The respiratory effects of optogenetic stimulation of C1 neurons

The respiratory effects of selective stimulation of C1 neurons in a Cre-transgenic mouse consist primarily of an increase in breathing frequency (f_R). For experimental details see (Abbott et al., 2013b). Breathing was measured using unrestrained whole-body plethysmography, upward deflections in flow trace indicate inspiratory efforts.

Figure 5. Optogenetic stimulation of C1 and RTN neurons causes arousal in sleeping rats

A. Response to stimulation of ChR2-expressing C1 and RTN neurons during non-rapid eye movement (NREM) sleep. Stimulation caused immediate and large cardiorespiratory effects shortly followed by desynchronization of the electrocorticogram (EEG) and appearance of neck muscle electromyographic (EMG) activity (i.e. arousal) that persisted throughout the stimulus. Arterial pressure (AP) was measured from femoral artery using radiotelemetry, breathing movements were measured using unrestrained whole-body plethysmography, upward deflection in flow indicates inspiration. Arrowhead indicates the first signs of EEG desynchronization; asterisk indicates an augmented breath (i.e. sigh) B. Group data showing the probability of arousal during stimulation in NREM sleep in rats injected with a Channelrhodopsin2 virus (ChR2⁺) vs. controls. C. As in A, except during rapid-eye movement (REM) sleep. Note the attenuated cardiorespiratory effects of photostimulation relative to (A). D. As in B, except during REM sleep. Note that the probability of arousal was less during REM sleep vs. NREM sleep in ChR2⁺ rats. For experimental details see (Abbott et al., 2013b).

Figure 6. Putative contribution of the C1 neurons to hypoxia-induced arousal

The C1 cells are powerfully activated by the carotid bodies and innervate a variety of stress-related structures and areas potentially involved in the regulating the state of vigilance in addition to the ventrolateral medulla. We propose that the C1 neurons contribute to asphyxia-induced arousal in two ways. One mechanism may be the activation of chest and cardiopulmonary feedbacks consecutive to the direct or indirect activation of the RPG. The second mechanism involves the direct activation of an inter-connected network of wake-promoting pathways (in blue) in the pons and hypothalamus by ascending projections of C1 cells (in orange). Known C1 targets include the locus coeruleus (LC), ventrolateral periaqueductal grey (vIPAG), IPB and the dorsomedial hypothalamus and perifornical region (DMH/Pf), which harbors orexinergic neurons (with projections shown in red). Arousal contributes in turn to enhancing the respiratory response by recruiting descending pathways.