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Medullary serotonin neurons and their roles in central respiratory chemoreception

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Abstract

Much progress has been made in our understanding of central chemoreception since the seminal experiments of Fencl, Loeschcke, Mitchell and others, including identification of new brainstem regions and specific neuron types that may serve as central "sensors" of CO_2/pH. In this review, we discuss key attributes, or minimal requirements a neuron/cell must possess to be defined as a central respiratory chemoreceptor, and summarize how well each of the various candidates fulfill these minimal criteria - especially the presence of intrinsic chemosensitivity. We then discuss some of the *in vitro* and *in vivo* evidence in support of the conclusion that medullary serotonin (5-HT) neurons are central chemoreceptors. We also provide an additional hypothesis that chemosensitive medullary 5-HT neurons are poised to integrate multiple synaptic inputs from various other sources thought to influence ventilation. Finally, we discuss open questions and future studies that may aid in continuing our advances in understanding central chemoreception.

1.0 Introduction

Breathing is tightly coupled to pH/CO_2, with hypercapnia providing a powerful respiratory stimulus. Changes in alveolar ventilation induced by a change in pH/CO_2 are initiated by biological sensors, or chemoreceptors, which are thought to be located in the central nervous system and in the carotid bodies. The majority of the chemoreceptor contributions to the ventilatory response to hypercapnia comes from the *central chemoreceptors* located in the brainstem, which possess the intrinsic ability to respond to changes in pH/CO_2, and through changes in cellular activity transduce an appropriate signal to components of the respiratory network to affect the output of the system. Currently, there is a great deal of focus on several specific brain regions (Putnam et al., 2004), and neurochemically-defined cell populations that might serve as central chemoreceptors (Pineda et al., 1997; Wang et al., 2001; Mulkey et al., 2004).

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Several observations derived from various approaches have given rise to the concept of multiple sites for central chemoreception, in which chemoreception is due to the combined effects of a number of brainstem nuclei acting collectively (Nattie et al., 2009). Perhaps the most powerful and widely accepted evidence for this theory comes from experiments using reverse microdialysis of high CO_2 solutions to generate regions of focal acidosis *in vivo*. In unanesthetized rats, focal hypercapnic acidosis within the nucleus of the solitary tract increases ventilation during sleep (by 16%) and wakefulness (28%) (Nattie et al., 2002), while focal acidosis in the RTN increases ventilation only during wakefulness (24%) (Li et al., 1999b), and in the medullary (midline) raphé only during sleep (20%) (Nattie et al., 2001b). Moreover, generating focal hypercapnic acidosis at multiple midline medullary raphé sites increases ventilation more than single sites in unanesthetized awake goats (Hodges et al., 2004a; Hodges et al., 2004b). While one can point to implicit assumptions and potential drawbacks with this approach (Guyenet et al., 2009), such as whether the stimulus is physiological or pathological, it seems likely that there are chemosensitive elements within these sites that drive ventilatory responses *in vivo*. But there are several remaining questions: What are the chemosensitive elements? Which components within these (and potentially other) anatomic locations can act as the sensors? Are they local cell bodies (neuronal or other), or dendrites or synaptic terminals from more distant chemosensitive neurons that account for these responses? There has been an assumption by many that the chemosensitive elements are neurons whose cell bodies are located in the region of interest. However, if they are instead synaptic terminals from neurons whose cell bodies are located distantly, that would radically change the interpretation of the results. Instead of widespread sites of chemoreception, the data could be interpreted as indicating that a limited number of cell types mediate chemoreception.

Our interest lies in determining what constitutes a chemosensitive element. The existing body of evidence suggests that the chemosensitive elements are cells (likely neurons) or parts of cells that possess an innate or intrinsic quality that confers upon them the ability to detect changes in pH/CO_2. Their intrinsic chemosensitivity combines with other extrinsic influences (synaptic or other inputs) to influence respiratory neurons and output under various conditions *in vivo*. We will review the evidence that specialized, intrinsically chemosensitive 5-HT neurons represent such a chemosensitive element.

In a series of recent reviews (Hodges et al., 2008a; Corcoran et al., 2009; Hodges et al., 2010), we have detailed much of the evidence supporting this conclusion, as well as the conclusion that 5-HT neurons provide tonic, excitatory input to the respiratory network. In this review, we revisit the minimal requirements that we previously suggested a central chemoreceptor must possess to be defined as such, including intrinsic chemosensitivity, and discuss why this requirement is important. We present *in vivo* data supporting a major role for 5-HT neurons in central chemoreception, and how this role is affected by intrinsic and extrinsic factors (synaptic inputs, arousal state). Finally, we discuss evidence suggesting that raphé 5-HT neurons are poised to integrate multiple other inputs that are important for breathing.

2.0 How should we define a central chemoreceptor?

As is apparent from the articles in this Special Issue, there are many groups of neurons that have been proposed to be central chemoreceptors. However, there are varying levels of evidence in support of each of these neurons playing this role. In order to come to any conclusion about this topic, we must first define the minimal requirements for a central chemoreceptor.

2.1 Central respiratory chemoreceptors: minimal requirements revisited

Despite progress in our understanding of the cellular mechanisms governing central chemoreception, our field is still trying to find consensus regarding one of the most fundamental issues - what are the criteria we use to define a neuron (or other cell type) as a central chemoreceptor? In two previous reviews, we defined a central *respiratory* chemoreceptor as cell that must have: 1) Intrinsic chemosensitivity to physiologically relevant changes in PCO_2, and; 2) The ability to induce appropriate effects on respiratory output (Richerson et al., 1998; Richerson et al., 2005). Here we apply this same definition but use the term central chemoreceptor, as it is implicit that we are concerned about chemoreceptors that alter ventilation. Before determining whether these criteria are met for a particular candidate, one has to first identify the specific subpopulation of cells that are putative chemoreceptors, rather than just identifying a nucleus, because identifying the specific neurons is necessary in order to definitively link data on intrinsic chemosensitivity, which can only be obtained *in vitro* (see below), with data demonstrating a functional effect on breathing, which can only be obtained *in vivo*. So far, this has only been done for three of the candidates: 5-HT neurons of the medullary raphé (Wang et al., 2001), Phox2b–expressing neurons of the retrotrapezoid nucleus (RTN) (Stornetta et al., 2006), and noradrenergic neurons of the locus coeruleus (LC) (Pineda et al., 1997). Until the specific neurons are identified in other regions, and they are proven to meet the minimal requirements for a chemoreceptor (see below), it will not be possible to prove whether those regions actually contain chemoreceptors. It is not enough to demonstrate that changes in pH in those regions induce changes in breathing, since the cellular elements that respond to pH could be dendrites or synaptic terminals from neurons with distant cell bodies.

2.2 Demonstrating intrinsic chemosensitivity

Another major issue for which there has not been consensus has been how to determine whether a neuron has intrinsic chemosensitivity. There have been many papers that have claimed to demonstrate intrinsic chemosensitivity using approaches that would not completely isolate neurons from extrinsic influences. Intrinsic chemosensitivity is particularly difficult to demonstrate, because there are many influences upon neurons other than fast synaptic transmission mediated by vesicular release of glutamate and GABA. There are many different neuromodulatory chemicals released at synapses by vesicular fusion (neuropeptides, monoamines, purines, etc.), and it is impossible to block all of the receptors that might be involved. It is also not well appreciated that vesicular neurotransmitter release is not completely blocked by removal of calcium [Need to add reference by Wu et al, J Neurophysiol, 2006] , or that there is non-vesicular release of many neurotransmitters. Some non-vesicular release is due to reversal of neurotransmitter

transporters (Levi et al., 1993; Wu et al., 2007), but there are other mechanisms of release, such as flux through hemichannels (Contreras et al., 2002), anion channels (Rutledge et al., 1998), and purinergic receptors (Sperlagh et al., 2002). In addition, many neurons are connected by electrical synapses, and there are many other mechanisms by which neurons and glia can influence each other, including gases such as NO and CO. Most papers claiming intrinsic chemosensitivity have used methods to block only one or two of these methods of intercellular communication, but there is no possible hope that all of them could be blocked *in vivo* or in brain slices. Tetrodotoxin (TTX) can block distant inputs, but does not prevent local interactions independent of action potentials, including most of the forms of communication described above. Physical isolation of a region by making brain slices or microislands is often used to isolate a region, but there is growing recognition that synaptic terminals may remain functional for 1–2 days after being separated from their cell body of origin. Given all of these possibilities, physical isolation of a cell is the only unequivocal way to eliminate all extrinsic influences. This method has its own limitations. For example, physical isolation causes damage to dendritic and axonal processes, which may prevent otherwise chemosensitive neurons from responding. However, unless this approach is used the conclusion cannot be made that a neuron has intrinsic chemosensitivity.

Attempts to test for intrinsic chemosensitivity have been made on the three phenotypically-identified chemoreceptor candidates. Medullary raphé 5-HT neurons display an unusually large increase (3-fold) in action potential firing rate in response to mild hypercapnic acidosis (pH 7.4 to 7.2) in acute brainstem slices and in primary cell culture *in vitro*. This cellular response to CO_2/pH in slices remains during conditions designed to reduce synaptic influences, such as high Mg^{2+}/low Ca^{2+}, or bath application of ionotropic glutamate and GABA receptors. The chemosensitive response of 5-HT neurons also persists in primary cell culture, where all synaptic connections from neurons outside the midline medullary raphé have been eliminated. These experiments demonstrate that intrinsic chemosensitivity is present in some type of cell within the raphé, but does not prove that 5-HT neurons themselves are the sensors. However, this has recently been shown, because medullary raphé 5-HT neurons retain a high degree of chemosensitivity after acute dissociation (Corcoran et al., 2009).

Similar to 5-HT neurons, the activity of specific neurons in the retrotrapezoid nucleus (RTN) correlate well with pH changes *in vivo* and in slice preparations *in vitro* (Mulkey et al., 2004). This relationship is maintained after application of a glutamate receptor antagonist applied to the surface of the brainstem *in vivo* (Mulkey et al., 2004). It is also not prevented by bath application of antagonists of purine, ionotropic glutamate, GABA and glycine receptors in acute slices, or after bath application of TTX in the neonatal *in vitro* brainstem-spinal cord preparation (Mulkey et al., 2007; Onimaru et al., 2008). These data have led some to conclude that these neurons are intrinsically chemosensitive. In a separate set of experiments, the pH response of RTN neurons was measured in the presence and absence of 5-HT in the bath (Mulkey et al., 2007). This caused a shift in the firing rate versus end tidal CO_2 curve to higher values, without a change in the slope. These data led to the conclusion that the pH response of RTN neurons is not due to synaptic input from nearby 5-HT neurons. However, the opposite conclusion could have been reached, since the

same result would be expected if chemosensitivity is due to synaptic input from 5-HT neurons. Bath applied 5-HT would simply be additive with synaptically-released 5-HT. The experiment that is needed to rule out this possibility is to measure chemosensitivity of RTN neurons after application of antagonists of 5-HT (and substance P) receptors.

Noradrenergic neurons of the LC have also been tested for intrinsic chemosensitivity (Pineda et al., 1997; Filosa et al., 2003; Putnam et al., 2004; Nichols et al., 2008). Like 5-HT and Phox2b^{+} RTN neurons, chemosensitive neurons in the LC are sensitive to hypercapnic acidosis using *in vitro* slice preparations under various conditions, including high Mg^{2+}/low Ca^{2+} solutions and ionotropic glutamate and GABA receptor blockade (Johnson et al., 2008; Nichols et al., 2008). Their response has also been tested before and after blockade of gap junctions with carbenoxelone (Nichols et al., 2008), which showed that electrical synapses account for some but not all of the neuronal responses in the LC.

While it is important to recognize that these and other data demonstrating the maintenance of chemosensitive responses to CO_2/pH after these various experimental manipulations are suggestive of an intrinsic chemosensory response of RTN and LC neurons, the limitations to each approach prevent firm conclusions. Data have not yet been published on chemosensitivity of either cell type after physical isolation.

The possibility has been raised that intrinsic chemosensitivity is a near-ubiquitous property of neurons distributed throughout the respiratory network, based in large part on a body of work first using microinjections of acetazolamide into various respiratory nuclei (Coates et al., 1993) and then later using focal acidosis *via* reverse microdialysis (Li et al., 1999a; Nattie et al., 2001a; Li et al., 2002; Nattie et al., 2002; Dias et al., 2008). In a recent paper (Erlichman et al., 2009), it was concluded that this possibility was supported by data using the voltage sensitive dye di-8-ANEPPS to measure mean membrane potential of neurons in brainstem slices. A reduced calcium/elevated magnesium solution was used “to reduce Ca^{++}-dependent synaptic transmission,” and neurons that continued to respond to hypercapnic acidosis were concluded to be “intrinsically CO_2 sensitive cells.” The magnitude of the optical response was then implicitly assumed to correlate with the size of their firing rate change. However, there are two flaws in the assumptions that led to these conclusions. First, there are many mechanisms of intercellular communication that are not blocked by reduced calcium/elevated magnesium solution (see above). Second, and equally importantly, a change in mean membrane potential does not necessarily correlate with a change in firing rate. For example, 5-HT neurons increase their firing rate 3-fold in response to acidosis without any change in mean membrane potential. This occurs because there is an increase in post-spike afterhyperpolarization with increased firing rate (Wang et al., 2001). Thus, although it remains a plausible hypothesis, the cellular evidence supporting the conclusion that intrinsically chemosensitive neurons are distributed widely throughout the respiratory network remains unconvincing.

The presence of intrinsic chemosensitivity does not automatically qualify a neuron as an important chemoreceptor. Some neurons may have a small intrinsic response to a large drop in pH, and a very large change in firing rate in response to mild hypercapnia *in vivo*. However, if the majority of their response *in vivo* is due to synaptic inputs from other

chemoreceptors then that neuron can not be viewed as the primary sensor under normal conditions.

2.3 Stimulation of respiratory output

As stated above, demonstrating intrinsic chemosensitivity alone does not qualify a central chemoreceptor as being capable of directly affecting ventilation. Thus, the second major criterion that must be met is demonstrating the ability to stimulate respiratory output in response to acidosis. This could occur if a neuron either projected to, or was embedded within, the respiratory network. Determining if a cell meets this criterion can only be accomplished if the specific subtype of cell is first identified. Of the three subtypes of phenotypically identified chemoreceptor candidates, this has only been clearly demonstrated in the case of glutamatergic RTN and raphé 5-HT neurons. For example, channelrhodopsin was selectively expressed in Phox2b^{+} RTN neurons *in vivo*, and light-induced stimulation increased baseline ventilation in anesthetized rats (Abbott et al., 2009). Neurotoxic (Nattie et al., 1991; Nattie et al., 1994; Takakura et al., 2008) or genetic (Dubreuil et al., 2008) lesions of the RTN lead to a decrease in respiratory output. Likewise, 5-HT neurons of the raphé obscurus project to and stimulate output of respiratory neurons in rostral medullary brain slices (Ptak et al., 2009), genetic deletion of 5-HT neurons reduces respiratory output in neonatal mice (Hodges et al., 2009), and antagonists of serotonin receptors reduce respiratory output in the *in situ* perfused brain of both neonatal and juvenile rats (Hodges et al., 2009; Ptak et al., 2009). Several studies suggest a role for the LC in ventilatory control (summarized in (Li et al., 2006)), though there seems to be less evidence for strong, direct projections from the LC to the respiratory network.

It is not possible to fulfill this criterion for chemoreceptor candidates that have not yet been phenotypically identified, because it is not known if a neuron can increase respiratory output without having a complete characterization of its projections, neurotransmitter content and other properties.

3.0 Supportive evidence for a role of 5-HT neurons as central chemoreceptors

In addition to meeting the two essential criteria for chemoreceptors, there is a considerable body of other data consistent with a role for 5-HT neurons in respiratory chemoreception.

3.1 Anatomical location of 5-HT neurons

The majority of medullary 5-HT neurons are located in and near the midline in the raphé nuclei. However, there is also a large subset whose location closely correlates with the classically defined rostral and caudal chemosensitive zones of the ventrolateral medullary (VLM) surface (Mitchell et al., 1963; Schlaefke et al., 1970) (Fig. 1A). In those early experiments, VLM 5-HT neurons may well have been the cell type that was stimulated by acidic solutions applied to the ventral medullary surface. During those experiments the investigators did not test the midline, because they wanted to avoid the large basilar artery, so they could not have detected the midline group of medullary 5-HT neurons. However, there are 5-HT neurons both in the midline and in the VLM, and in both cases they are

closely associated with large blood vessels on the brainstem surface (Fig. 1B). The majority of those that are deep to the surface are also close to large penetrating arteries (Bradley et al., 2002). In these perivascular locations, 5-HT neurons would be ideally suited to faithfully detect changes in blood PCO_2 before it is influenced by brain metabolism. The PCO_2 in this location, better than anywhere else in the CNS, would closely reflect lung ventilation. There is not necessarily an advantage to detect changes in blood PCO_2 rapidly, but there is an advantage to measure it accurately.

3.2 5-HT neuron activity *in vivo*

5-HT neurons fire action potentials in a highly-regular, pacemaker-like manner. This pattern and the firing rate of 5-HT neurons are remarkably constant in behaving animals during a variety of behaviors and experimental manipulations, and throughout the brainstem raphé nuclei (Jacobs et al., 2008). This is true even during changes in homeostatic variables that 5-HT neurons are thought to influence, such as sympathetic activity. 5-HT neuron firing rate was shown to be unaffected by elevated environmental temperature or pharmacologic "fever", tonic or phasic painful stimuli, acoustic stimuli, physical restraint, exposure to a natural predator, pharmacologically-induced changes in blood pressure, and insulin-induced hypoglycemia (Auerbach et al., 1985; Fornal et al., 1987; Jacobs et al., 1992; Martin-Cora et al., 2005).

While 5-HT neuron activity in general is remarkably unaffected by most behaviors and/or experimental manipulations, their activity is highly correlated with the arousal state of the animal (Jacobs et al., 1991). Firing rates of 5-HT neurons throughout the B1–9 nuclei are highest during active wakefulness, lower during non-rapid eye movement (NREM) sleep, and lowest or quiescent during REM sleep. Moreover, the activity of many 5-HT neurons is also increased with specific repetitive motor activities. For example, virtually all medullary 5-HT neurons are activated during treadmill locomotion (Veasey et al., 1995). However, these neurons do not all behave the same, and specific subpopulations appear to be selectively activated by specific behaviors or conditions. For example, 25% of dorsal raphé nucleus (DRN), and 22% of medullary 5-HT neurons increase firing rate during CO_2 breathing, with significant activation detected in response to as little as 3% inspired CO_2 (Veasey et al., 1995; Veasey et al., 1997). The magnitude of the neuronal response of some of these neurons is directly correlated with ventilatory motor output. Interestingly, the neuronal responsiveness to elevated inspired CO_2, like the ventilatory response of the animal, is reduced during sleep. There is also a subset (25%) of 5-HT neurons in the DRN that increase activity (up to 5-fold) before the initiation of chewing behavior, and the increased activity halts coincidentally with the cessation of the behavior (Fornal et al., 1996). Overall, these data show that the activity of 5-HT neurons *in vivo* is relatively resistant to most experimental manipulations, but is highly correlated to arousal state and various repetitive motor activities, including breathing during hypercapnia. The common link between these types of motor behavior is unclear, but has been interpreted as demonstrating a role of these neurons in repetitive motor patterns (Jacobs et al., 1992). [I disagree that putting a statement about feedforward stimulation of breathing during exercise is somehow a dangerous thing. It is just conjecture that would get people thinking about this

possibility. This type of sentence is quite common and a good way to advance thinking. But if you agree that somehow it is “unsafe” then go ahead and take it out.]

There is a large body of additional data confirming that a subset of 5-HT neurons increases their firing rate during hypercapnia *in vivo*. For example, microdialysis experiments have shown that 5-HT release in the hypoglossal motor nucleus is increased in response to inhalation of 5, 7, and 9% CO_2 in mice (Kanamaru et al., 2007). Numerous papers have also shown that c-fos is activated in medullary raphé neurons in response to hypercapnia *in vivo*, including those shown to be immunoreactive for 5-HT or tryptophan hydroxylase (Larnicol et al., 1994; Haxhiu et al., 1996; Haxhiu et al., 2001; Pete et al., 2002; Johnson et al., 2005). c-fos is activated in response to hypercapnia in cells on the rat brainstem surface in a distribution that closely correlates with 5-HT neurons of both the VLM and the midline (Fig. 1C). Interestingly, it has also been found that ATP is released in response to hypercapnia *in vivo* on the medullary surface, in a distribution that also closely correlates with the location of 5-HT neurons (Fig. 1D) (Gourine et al., 2005). The relationship between this ATP release, thought to occur from glia, and the chemosensitivity of 5-HT neurons is unclear.

3.4 Inactivation, lesions and genetic studies

As summarized in two recent reviews, there is additional *in vivo* data obtained using a variety of experimental approaches that are consistent with a major role for the medullary raphé, and more specifically 5-HT neurons, in central respiratory chemoreception. These approaches include general or specific chemical lesions of raphé/5-HT neurons and genetic deletion of most or all central 5-HT neurons in transgenic mice.

Medullary raphé injections of the excitatory neurotoxin ibotenic acid (IA), which is thought to selectively kill neurons whose cell bodies are nearby, have effects on ventilation and CO_2 chemoreception in both the anesthetized and awake states. For example, IA injections into the medullary raphé of anesthetized or decorticate piglets reduced both the phrenic and hypoglossal responses to increased CO_2 (Dreshaj et al., 1998). A more dramatic effect of IA injections into the ventral medullary raphé has been seen in anesthetized rats, with animals initially showing tachypnea and arterial hypoxemia and acidosis, followed by terminal apnea within 90 minutes of injection in all rats (Carruth et al., 1992). IA injections into the medullary raphé of awake goats also caused acute hyperpnea, followed by transiently reduced CO_2 sensitivity (Hodges et al., 2004c; Hodges et al., 2005b).

More selective neuronal lesions targeting 5-HT neurons have also consistently decreased the systemic response to hypercapnia, and in some cases led to hypoventilation. For example, targeted destruction of midline medullary 5-HT neurons using a saporin-conjugated antibody targeting the serotonin transporter (SERT) depresses the ventilatory response to hypercapnia, but the degree of neuronal loss (~31%) and attenuation of the CO_2 response (15–18%) were both small (Nattie et al., 2004). 5-HT neuron lesions by intracerebroventricular 5,7-DHT administration had far more dramatic effects, reducing baseline respiratory output and the slope of the relationship of ventilation to arterial PCO_2 during anesthesia (Mueller et al., 1985).

Perhaps the most powerful supportive evidence for a major role for 5-HT neurons in central chemoreception is from the study of transgenic knockout mice with 5-HT system dysfunction (discussed in detail in a recent review (Hodges et al., 2010)). Conditional deletion of the *Lmx1b* gene in all *Pet1*-expressing neurons leads to near-complete (>99%) loss of 5-HT neurons in the CNS (Zhao et al., 2006). This loss is selective for 5-HT neurons, whereas other monoamine systems, and general histological appearance remained unaltered. As adults, $Lmx1b^{f/f/p}$ mice breathe relatively normally at rest and while breathing a hypoxic gas mixture (Hodges et al., 2008b). However, these mice display more than a 50% reduction in the hypercapnic ventilatory response. In addition, the hypercapnic ventilatory response could be augmented with increased central 5-HT (delivered intracerebroventricularly), with the highest levels of infused 5-HT restoring the response to hypercapnia to near wild type levels. The recovery of the hypercapnic ventilatory response with exogenous (intracerebroventricular) 5-HT suggests that 5-HT can either: 1) increase the response of the respiratory network to input from peripheral and/or other central chemoreceptors, or; 2) enhance the CO_2 response of other central chemoreceptors (Hodges et al., 2008b; Corcoran et al., 2009). Data from transgenic mice with other forms of 5-HT system dysfunction also display selective deficits in the hypercapnic ventilatory response (Hodges et al., 2005a; Li et al., 2008), consistent with a major role for 5-HT neurons in central respiratory chemoreception *in vivo*.

4.0 Beyond intrinsic chemosensitivity – extrinsic effects on chemoreception

As stated above, intrinsic chemosensitivity *in vitro* does not eminently qualify a group of neurons/cells as being important *in vivo*. Even if a neuronal population is intrinsically chemosensitive and capable of driving changes in alveolar ventilation, their relative contributions may be conditional or state-dependent in the intact, unanesthetized animal. Moreover, a chemosensitive neurons' ability to sense changes in CO_2/pH is not static, and is likely modulated by a number of other factors. For example, the degree of chemosensitivity of 5-HT neurons is age and state-dependent. These neurons begin to display cellular chemosensitivity to hypercapnic acidosis around post-natal day 12 (P12), and do not exhibit a robust (mature) CO_2 response until after P20 *in vitro* (Wang et al., 1999; Wu et al., 2008). This pattern of chemosensitivity parallels the development of the whole-animal response to hypercapnia *in vivo* (Stunden et al., 2001; Davis et al., 2006). In contrast, other chemoreceptor candidates do not show the same developmental maturation, with chemosensitivity being present at birth (Stunden et al., 2001; Ritucci et al., 2005; Conrad et al., 2009; Dubreuil et al., 2009).

In vivo data demonstrate that the firing rate of 5-HT neurons and the degree of chemosensitivity to inhaled CO_2 is highly correlated with the arousal state of the animal, where 5-HT neuron activity and chemosensitivity are higher during wakefulness compared to sleep (Veasey et al., 1995; Veasey et al., 1997), providing evidence for the influence of extrinsic factors altering cellular chemosensitivity. Another example is the report of minimal chemosensitivity of medullary 5-HT neurons recorded in halothane-anesthetized rat preparations *in vivo* (Mulkey et al., 2004), which suggests that in the anesthetized state,

extrinsic factors blunt expression of a chemosensory response in 5-HT neurons. This may be due to activation of TASK currents by halothane, which would mask any effect of CO_2/pH by clamping the membrane potential due to a high conductance state. Therefore, age, arousal state and anesthesia are examples of factors that modify and shape the role of central respiratory chemoreceptors *in vivo*.

5.0 Interactions of the raphé with other putative chemoreceptor sites

While there is continued focus on the neurons responsible for acting as the sensor (those intrinsically chemosensitive to CO_2/pH), the importance of synaptic events in determining the response of the respiratory control system as a whole cannot be diminished. [I took out the Leiter, 2009 reference because I think it is a terrible paper, and it is definitely not the first or best paper to make this point.] It is likely that there are some neurons that contribute greatly to maintaining eupneic PCO_2, or to the behavioral response to CO_2/pH (chemoreflex) as a point of chemosensory integration, or a site of chemosensory signal amplification, despite minimal or no intrinsic chemosensitivity.

A case can be made for the medullary raphé as a site of integration for chemosensory and multiple other afferent inputs. Medullary raphé (obscurus and pallidus) neurons receive afferent inputs from the hypothalamus (including the dorsal, dorsomedial, lateral and posterior hypothalamic areas), medial preoptic area, multiple subdivisions of the periaqueductal grey, the parabrachial and Kölliker-Fuse nuclei, the nucleus of the solitary tract, and locus coeruleus (Aghajanian et al., 1977; Morrison, 2004; Verner et al., 2008). There are reciprocal connections between raphé obscurus 5-HT neurons and pre-Bötzinger Complex neurons (Ptak et al., 2009). In addition, it has recently been demonstrated that subpopulations of raphé neurons are functionally connected to pontine and ventral respiratory groups (Nuding et al., 2009), are influenced by peripheral chemoreceptor stimulation (Morris et al., 1996a; Morris et al., 1996b; Nuding et al., 2009), and are affected by alteration or absence of pulmonary stretch receptor feedback (Morris, 2007). Although it was not determined if the raphé neurons that receive these many afferent synaptic inputs are chemosensitive and/or serotonergic, the possibility remains that raphé 5-HT neurons may also serve as a point of integration of chemosensory and other afferent synaptic inputs.

Similarly, the collective evidence also suggests a major role of RTN neurons in modulating eupneic ventilation, and chemosensory integration (Nattie, 2006; Guyenet et al., 2009). Chemical lesions of the RTN region using excitatory neurotoxins have large effects on baseline ventilation and CO_2 sensitivity (Nattie et al., 1991; Nattie et al., 1994; Akilesh et al., 1997), and reversible neuronal dysfunction of the ventrolateral medullary surface in this region leads to hypopnea during wakefulness, and reversible or terminal apnea during sleep or anesthesia, respectively (Ohtake et al., 1995; Forster et al., 1997). Selective lesions of NK-1 receptor-expressing RTN neurons increase the apneic threshold (Takakura et al., 2008), although these same lesions increase the slope of the relationship between ventilation and end-tidal CO_2 (the opposite of what would be expected if the RTN is a site of chemosensitivity). Moreover, recordings from RTN neurons in anesthetized rats demonstrate direct activation by peripheral chemoreceptor stimulation (Takakura et al., 2006), whereas lung stretch receptor activation inhibits the activity of CO_2-responsive $Phox2b^+$ RTN

neurons (Moreira et al., 2007), and stimulation of the posterior hypothalamus increases RTN neuronal activity (Fortuna et al., 2009). Finally, exogenous (and presumably endogenous) 5-HT and substance P activate CO_2-sensitive RTN neurons, suggesting that chemosensitive 5-HT neurons may also stimulate the RTN. Thus, both the medullary raphé and RTN nuclei are putative central chemoreceptors, provide excitatory drive to the respiratory network, and appear to be equally poised to integrate multiple synaptic inputs. This suggests that these nuclei may both represent "crucial nodal point[s] through which breathing automaticity is regulated to maintain CO_2 constant" (Guyenet et al., 2009).

6.0 Summary and Conclusions

Consensus is still lacking on the definition of the critical attributes that a neuron (or cell) must possess to be accepted as a central respiratory chemoreceptor, although this would help the field advance beyond current debates. Satisfaction of the two essential criteria defined here requires a combination of approaches, from the highly reduced, physically isolated neuron to the intact, unanesthetized animal. New discoveries could drive more concrete conclusions. In the meantime it will remain important to think critically about the strength of the evidence in favor of each chemoreceptor candidate, and in particular whether they possess an intrinsic response. If they do have intrinsic chemosensitivity, it will then be important to determine the relative contribution of each one to the normal ventilatory response, and how this contribution varies under different conditions due to influences of various extrinsic factors.

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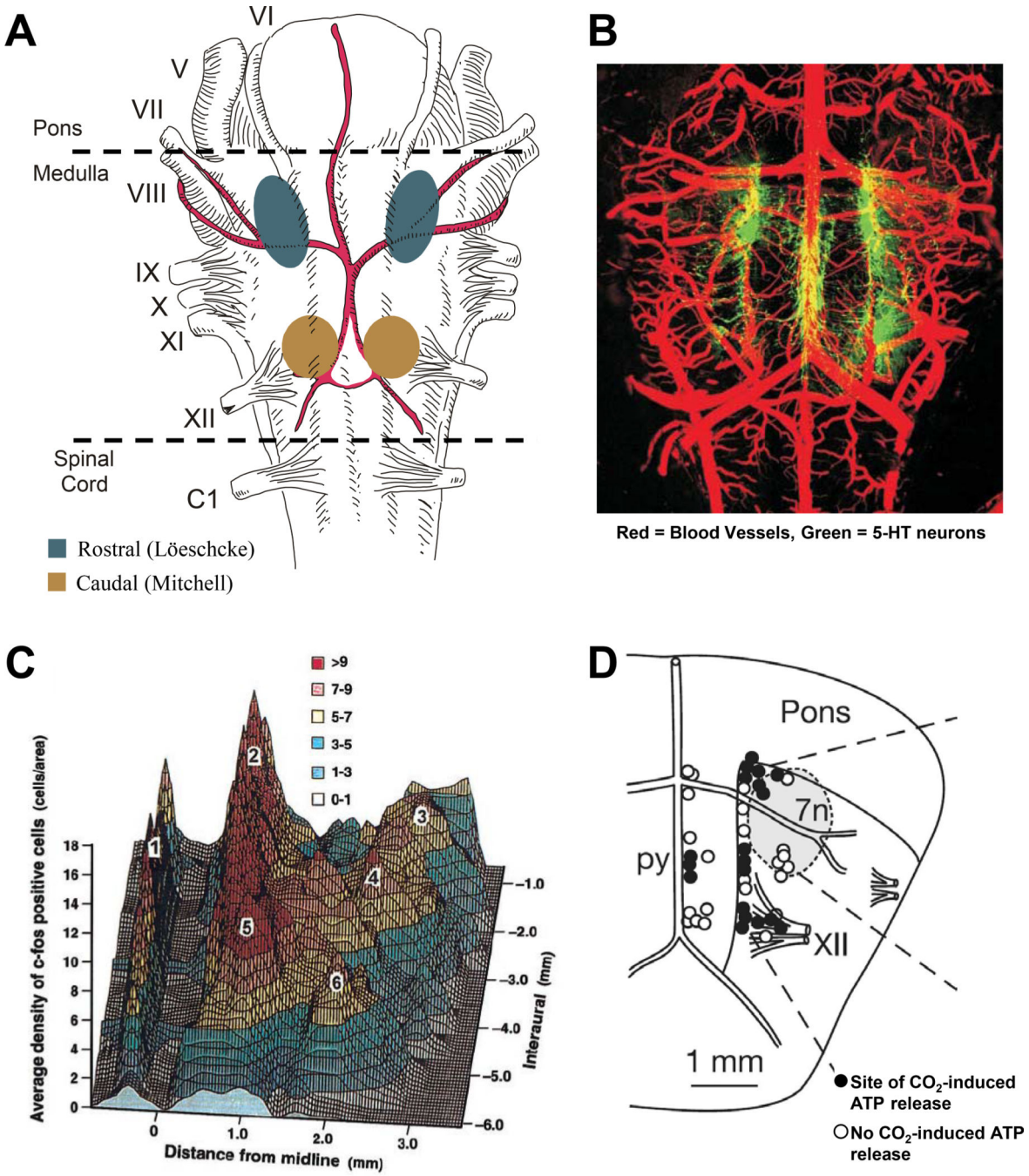

Figure 1.

(A) A sketch representing the estimated location(s) of the rostral (blue) and caudal (tan) chemosensitive zones originally described by Mitchell and Löeschcke, respectively. (B) Confocal images of blood vessels on the surface of the brainstem superimposed onto the midline and ventrolateral distribution of 5-HT neurons. Note that in addition to those on the midline, there are also ventrolateral areas with a high density of 5-HT neurons, underlying the classically-described chemosensitive regions. (C) There are c-fos positive neurons in the midline and on the ventral medullary surface of animals exposed to progressive hypercapnic

acidosis (5, 7, and 9% CO_2) that are in the same regions as 5-HT neurons. (D) A sketch of the ventral medullary surface indicating areas probed that displayed (solid circles) or did not display (open circles) hypercapnia-induced ATP release. These also correspond to the location of 5-HT neurons in both the midline and VLM. (A) Adapted from the Handbook of Physiology (Feldman, 1986). (B) Adapted from Bradley et al., Nat. Neurosci., 5(5): 401–2, 2002. (C) Adapted from Okada et al., J. Appl. Physiol. 93(2): 427–39, 2002. (D) Adapted from Spyer at al., Nature 436(7047): 108–11, 2005.