Published in final edited form as:

Respir Physiol Neurobiol. 2010 October 31; 173(3): 298–304. doi:10.1016/j.resp.2010.03.022.

Ionic mechanisms of central CO₂ chemosensitivity

Mykyta M. Chernov¹, Joseph S. Erlichman², and J.C. Leiter¹

¹Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756

²Department of Biology, St. Lawrence University, Canton, NY 13617-1475

Abstract

A comparative analysis of chemosensory systems in invertebrates and vertebrates reveals that different animals use similar strategies when sensing CO₂ to control respiration. A variety of animals possess neurons that respond to changes in pH. These respiratory chemoreceptor neurons seem to rely largely on pH-dependent inhibition of potassium channels, but the channels do not appear to be uniquely adapted to detect pH. The ‘chemosensory’ potassium channels identified thus far are widely distributed, common potassium channels. The pH-sensitivity is a common feature of the channels whether the channels are in chemosensory neurons or not. Thus, the pattern of synaptic connectivity and the mix of potassium channels expressed seem to determine whether a neuron is chemosensory or not, rather than any special adaptation of a channel for pH-sensitivity. Moreover, there are often multiple pH-sensitive channels in each chemosensory neuron. These ionic mechanisms may, however, be only part of the chemosensory process, and pH-dependent modulation of synaptic activity seems to contribute to central chemosensitivity as well. In addition, the exploration of the mechanisms of pH-dependent modulation of ion channel activity in chemosensory cells is incomplete: additional mechanisms of pH modulation of channel activity may be found, and addition conductances, other than potassium channels, may participate in the chemosensory process.

1. Introduction

In 1982, H.H. Loeshke published a review titled, ‘Central Chemosensitivity and the Reaction Theory’ (1982). The work represented the state of the art in studies of central chemosensitivity, and it put forward three theses: first, the apparent chemosensory stimulus was extracellular pH and the proton rather than molecular CO₂; second, the central chemosensory sites were on the ventral surface of the medulla and there were rostral and caudal chemosensory sites plus an intermediate integrative site; and third, the central chemosensory mechanism involved proton-dependent enhancement of acetylcholine activity at synapses within the central chemosensory areas. This paper represented the culmination of more than 40 years of research that started when Gesell and others recognized that hypercapnia acted as if it increased the potency of acetylcholine, apparently by decreasing the activity of acetylcholine esterase (Gesell and Hansen, 1943). Thus, the central chemosensory mechanism was ventrally located and dependent on extracellular pH within acetylcholinergic synapses. The paper was a watershed event in that, since that time,

© 2010 Elsevier B.V. All rights reserved.

Corresponding author: J.C. Leiter, Dept. of Physiology, Dartmouth Medical School, Lebanon, NH 03756, Tel: 603-650-8533, Fax: 603-650, james.c.leiter@dartmouth.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

virtually the entire focus on the mechanisms of central chemosensitivity has shifted to intrinsic ionic mechanisms within the neuronal cell membrane (rather than synaptic processes) regulated by intracellular pH (rather than extracellular pH) in locations that do include the ventral medulla, but extend throughout the brain from the hypothalamus to the dorsal, ventral and caudal medulla. Therefore, we will discuss current views of the central chemosensory stimulus and central chemosensory mechanisms involving ion channels in invertebrates and mammals. In mammals, we will discuss a pontine chemosensory site, the locus coeruleus, and three medullary sites: the retrotrapezoid nucleus (which includes at least the rostral area studied by Loeschke), the nucleus of the tractus solitarius and the caudal raphé.

2. The chemosensory stimulus: intracellular or extracellular pH

The most general view of chemosensory processes entails protons interacting with voltage- or ligand-gated channels in the cell membrane of neurons to increase or decrease the activity of each neuron. Presumably, the protons interact with titratable amino acid residues, and chemosensitivity is imparted by changes in ion channel kinetics – the channel conductance, the open probability, the activation or inactivation characteristics or time dependence of voltage-gated channels, or the affinity of binding or conductance characteristics of ligand-gated channels. These ideas are entirely consistent with the alpha-stat hypothesis (Rahn et al., 1975; Reeves, 1972), which posits that animals regulate their pH to maintain a constant charge state of imidazole residues in histidine. Histidine is a ubiquitous amino acid, and the imidazole residue has a pKa close to the normal physiological pH. The relative charge state of imidazole varies as a function of pH and so alters the conformation and function of the proteins in which they exist to generate a chemosensory response (Somero, 1986). Imidazole imparts pH sensitivity to many ion channels, but any titratable group with a pKa in the physiological range might do. For example, one of two cysteines seem to be responsible for pH sensitivity in the inwardly rectifying potassium channel, HIR, but the pKa of these cysteines is brought into the physiological range by coordination with a histidine residue (Coulter et al., 1995). However, histidine need not be involved at all if other biophysical characteristics of the channel bring the pKa of titratable amino acids into the physiological range. Hydrophobicity in the region of the selectivity filter of the two-pore domain channel, TASK2, seems to allow an arginine to determine pH-sensitivity in this channel (Niemeyer et al., 2007).

Titratable amino acid groups may be present at the extracellular or intracellular surface of proteins in the cell membrane, and it would be surprising if changes in proton concentration in either the extracellular or intracellular space did not alter the function of chemosensory proteins. However, intracellular pH is a sufficient and important chemosensory stimulus in an air breathing invertebrate (Erllichman and Leiter, 1994) and intracellular pH seems to be the dominant stimulus for electrophysiological responses in central chemosensory cells in mammals (Filosa et al., 2002; Wang et al., 2002). Intracellular pH appears to be a sufficient chemosensory stimulus in mammalian neurons, but it is not a necessary chemosensory stimulus – the frequency response of chemosensory neurons was not a unique function of intracellular pH, and changes in intracellular pH, intracellular calcium or as yet unidentified factors may modify the neuronal response to hypercapnia (Filosa et al., 2002; Putnam, 2010; Wang et al., 2002). The entire discussion of whether intracellular or extracellular pH is the dominant chemosensory stimulus may be moot when hypercapnia is the stimulus. The buffer capacity of chemosensory neurons has been rather low when measured (Goldstein et al., 2000; Ritucci et al., 1998; Ritucci et al., 1997). As a consequence, the change in intracellular and extracellular pH is about the same for any change in the common physiological stimulus – CO₂. Since CO₂ readily diffuses across cell membranes, either intracellular or extracellular pH provides a reasonably similar report of changes in the local pH

environment. Although extracellular and intracellular pH may vary in parallel locally, there is surprising heterogeneity of extracellular (and presumably intracellular) pH regionally within the brainstem (Arita et al., 1989). Therefore, the pH environment among different chemosensory sites may be quite variable for equivalent levels of tissue CO₂ by virtue of local neuronal factors or the differential actions of astrocytes in specific chemosensory areas of the brain (Erllichman and Leiter, 2010).

3. Putative ion channels regulating neuronal activity in central chemosensory neurons

The activity of neurons may be modified by varying the level of the resting membrane potential to hyperpolarize the neuron (and diminish its activity) or depolarize the neuron to its threshold for excitation and increase its activity. The resting membrane potential is dominated by potassium currents and so most of the putative chemosensory ion channels are potassium channels. The most general mechanism proposed involves pH-dependent inhibition of those channels that are open at the resting membrane potential. Inhibition of these channels increases neuronal activity, and if the activity of the channels is modulated by pH, the change in neuronal activity will also be pH-dependent. Most studies of central chemosensitivity emphasize the role of neurons excited by hypercapnia. Hyperpolarization of neurons is clearly a chemosensory response, but the ion channels responsible for pH-dependent hyperpolarization of neurons have not received much attention and will not be reviewed.

Identification of putative pH-sensitive ion channels in chemosensory cells relies on two methods, both of which have limitations. Neurons in chemosensory areas may be studied electrophysiologically under current clamp conditions in which one observes the shape of the action potential and uses a variety of inhibitors to remove the effects of particular channels and then infers from the response whether the inhibited channel contributed to hypercapnic responses. The inhibitors are not as specific as one might wish, and so the involvement of the channel can only be tentatively identified. The alternate strategy is to use voltage clamp and study individual currents using combinations of inhibitors and voltage clamp protocols that isolate currents associated with specific channels based on activation or inactivation characteristics. One tends to obtain more information about specific channels and their kinetic characteristics in voltage clamp studies, but these studies are limited by the inhibitors available and by the fact that it is difficult to clamp the voltage of entire cells when they have extensive arborizations. As a result, voltage clamp studies are dominated by the effects of somatically expressed channels; the function of pH-sensitive channels expressed only in the distal dendritic tree will be difficult to identify under voltage clamp conditions. Notwithstanding these limitations, considerable progress has been made identifying pH-sensitive channels in a variety of chemosensory sites using electrophysiological and pharmacological methods in wild-type and transgenic animals.

4. Invertebrate CO₂ sensitivity: insects

The respiratory and feeding behaviors of many insects are elicited by or depend on CO₂ chemoreception. There are, so far as we know, no studies of the ionic basis of spiracular control by CO₂. On the other hand, many hematophagous insects are sensitive to the odor of CO₂. Mosquitoes, for example, sense CO₂ expired by their prey and follow the CO₂ gradient to locate and bite their intended host. Similarly, the herbivorous hawkmoth, *Manduca sexta*, uses the amount of CO₂ given off by flowers to evaluate their nectar content (Thom et al., 2004). Fruit flies sense CO₂ as well, but in them, it elicits an avoidance reaction. The odorant CO₂ sensors expressed in the antennal organs of fruit flies have recently been identified. CO₂ chemoreception in the fruit fly antennae is mediated by a heterodimer

formed from two seven-transmembrane-domain proteins, Gr21a and Gr63a, which are members of the gustatory receptor gene family (Jones et al., 2007; Kwon et al., 2007). The nature of the stimulus (whether it is molecular CO₂ or pH) remains unknown. Gustatory receptors in insects are generally odor-gated ion channels, and the CO₂ sensitive odor channels are uniquely expressed in the CO₂ sensitive neurons in *Drosophila*. Thus, CO₂ sensitivity in insects seems to be associated with highly specialized, unique sets of channels tuned to pH or molecular CO₂. The strategy for creating respiratory CO₂ chemosensors is completely different; respiratory CO₂ sensitivity in mammals seems to rely on the pH-sensitivity of widely expressed and common potassium channels (see below) – no unique or highly specific CO₂ sensitive channels dedicated to respiratory control have been identified in vertebrates thus far.

4.1 Invertebrate CO₂ sensitivity: pulmonate snails

Respiration in pulmonate snails is controlled by regulating the access of air to the lung via a sphincter, the pneumostome. Elevated CO₂ (3–5%) increases opening of the pneumostome (Dahr, 1924). The pneumostomal response to hypercapnia originates centrally from a small number of neurons in the subesophageal ganglia (Erichman and Leiter, 1993), and the chemosensory response is correlated with intracellular, rather than extracellular, pH (Erichman and Leiter, 1994). Current clamp studies in a closely related species, *Helix pomatia*, indicated that only a subset of CO₂ sensitive neurons within the subesophageal ganglia controlled pneumostomal opening, even though elevated CO₂ altered the activity of many more neurons in the same ganglia (Erichman and Leiter, 1997). The activity of these respiratory chemosensory neurons was correlated with the CO₂ stimulus and correlated with pneumostomal activity. Hyperpolarization of individual neurons, despite persistent hypercapnia, reduced pneumostomal opening. This ability to obtain a clear physiological response by manipulating a single cell has not been replicated in the vertebrate CNS where stimulus and response is coded by populations of cells in which no single cell makes a clearly detectable contribution.

The dependence of neuronal activity on pH in snail chemosensitive neurons is mediated by potassium ion channels. Three potassium channels, whose maximal conductances and gating properties were affected by pH, were identified using voltage clamp protocols and pharmacological inhibitors of potassium channels in neurons isolated from the subesophageal ganglia (Denton et al., 2007). The three pH-sensitive conductances could be ascribed to expression of delayed rectifier (K_{DR}), fast A-type (K_A) and calcium-activated potassium channels. The maximal conductance of the channels was inhibited by low extracellular pH for all three types, and the K_A channel was inhibited by intracellular pH. The effects of extracellular pH on potassium channel function were a surprise since the behavioral response to CO₂ seemed to depend exclusively on intracellular pH (Erichman and Leiter, 1994). A computer model of a chemosensory neuron derived from the electrophysiological studies of *Helix aspersa* confirmed that the A-type channel is the primary determinant of the chemosensory neuron's firing rate, whereas the delayed rectifier channel and the calcium-activated potassium channel changed the shape of individual spikes (K_A and K_{DR}) and mediate the transition between various firing patterns such as spiking or bursting (the calcium-activated potassium channel is particularly important limiting burst duration) (Chernov et al., 2007). The important finding from these studies was the way in which the activity of multiple pH-sensitive potassium channels was coordinated to shape the neuronal response; respiratory chemosensitivity of these neurons did not arise from the presence of a single unique channel, but from interactions of multiple, common pH-sensitive potassium channels, all expressed in a single neuron.

5. Mammalian central CO₂ chemosensory neurons

Just as the putative stimulus and the mechanism of chemosensitivity have evolved since Loeschke formulated his views in 1982, so the location of respiratory central CO₂ chemosensory sites has evolved. There appear to be multiple sites of central chemosensitivity throughout the brainstem (Coates et al., 1993; Feldman et al., 2003) rather than sites solely on the ventral surface of the medulla. The ionic mechanisms that sense pH in or near chemosensory neurons seem to differ among sites. However, there have not been many electrophysiological studies of the mechanism of CO₂ sensitivity in individual neurons and certainly not many voltage clamp studies that would allow firm identification of the pH-sensitive channels active in specific cells. We will discuss four chemosensory nuclei in the brainstem: locus coeruleus, nucleus of the solitary tract, retrotrapezoid nucleus (which is probably the rostral chemosensory area studied by Loeschke), and the caudal raphe. These sites demonstrate the diversity of putative chemosensory channels identified thus far.

5.1 Chemosensory neurons in the locus coeruleus

Over 80% of neurons in the LC respond to hypercapnia (Putnam 2010). Chemosensitivity is conferred by modulation of multiple pH-sensitive channels, very much like CO₂ sensitivity in the pulmonate snail, although the channels involved differ somewhat. The first pH-modulated channel described was an inwardly-rectifying polyamine-sensitive potassium current (K_{IR}), which was inhibited by hypercapnia (Pineda and Aghajanian 1997). Ammonium prepulse experiments showed that the neurons were responding to changes in intracellular pH. Intracellular acidification also inhibited a TEA-sensitive potassium channel, and TEA sensitivity is typical of delayed rectifier potassium channels (Filosa and Putnam, 2003). More recently, a 4AP-sensitive potassium channel has been described in LC neurons (Putnam, 2010), and 4AP sensitivity is typical of A-type potassium currents. After TEA-treatment, LC neurons still expressed some residual pH-sensitive current (Filosa and Putnam, 2003). TASK channels are expressed in LC neurons, and so a TASK channel or TASK-like channel may also be active in LC neurons, but voltage clamp studies have not been conducted in LC neurons to prove this. In addition, knockout mice lacking TASK1 or TASK 3 or TASK1 and TASK3 had normal ventilatory responses to CO₂ when studied between the ages of 4–8 months (Mulkey et al., 2007b). Therefore, if a TASK channel plays a role in the LC, it must be some non-TASK1 or 3 TASK channel or some other TASK-like channel. Alternative possibilities are that there is sufficient redundancy within chemosensory LC neurons or among chemosensory sites to mask the loss of the TASK1 and TASK3 channels or the contribution of TASK channels to LC chemosensitivity is small.

Intracellular acidification of LC neurons also activated an L-type calcium channel, which seemed to contribute to neuronal chemosensitivity (Filosa and Putnam, 2003). Proton-dependent activation of calcium channels is very unusual; the typical effect of decreased pH on calcium channels is direct proton-mediated inhibition of the channel. For this reason, Putnam (2010) has suggested that excitatory pH effects on L-type calcium channels are not direct effects of pH on the channel, but originate indirectly from the increased bicarbonate (rather than the protons) associated with hypercapnia, and increased bicarbonate may activate soluble guanylate cyclase and thereby enhance calcium channel activity.

5.2 Chemosensory neurons in the NTS

The electrophysiological basis of CO₂ sensitivity has been less well studied in the NTS. The earliest studies indicated that a pH-sensitive, 4AP-sensitive A-type potassium channel was present in CO₂-sensitive NTS neurons (Dean, 1990; Dean et al., 1989). There has been no follow-up of these studies and no voltage clamp work on CO₂-sensitive neurons in the NTS. It is possible that a single ion channel determines pH-sensitivity in NTS neurons, but that

seems unlikely given the ubiquity of pH effects on ion channels and the morphology of the wave form of the action potential in NTS neurons, which cannot be explained by the activity of a single species of A-type potassium channel.

5.3 Chemosensory neurons in the RTN

The ventrolateral and rostral medullary location of the RTN suggests that this nucleus contains the rostral chemosensory neurons identified as area M in earlier experiments using topical application of acidic pledgets on the ventral medulla of the cat to identify chemosensory sites (Mitchell 1963). Characterization of the chemosensory neurons within the RTN has shown that the majority of pre-inspiratory neurons in the RTN/Parafacial region of the neonatal rat express the *Phox2b* gene (Onimaru et al., 2008; Stornetta et al., 2006). Mutations of the *Phox2b* gene in humans are responsible for Congenital Central Hypoventilation Syndrome (CCHS), a condition marked by the absence of a ventilatory response to hypercapnia and sleep apnea of central origin, among other disorders of autonomic nervous system function (Amiel et al., 2003). Genetically-engineered homozygous *Phox2b* mice die *in utero* (Pattyn et al., 2000). Therefore, heterozygous knockout or transgenic animals have been created to study the pathogenesis of CCHS. Heterozygous *Phox2b* null mice are relatively insensitive to CO₂ in the first week of life, but these animals recover normal hypercapnic sensitivity by post-natal day 10 (Gallego and Dager, 2008). On the other hand, mice heterozygous for a transgene for a 7 member alanine expansion in a 20 member polyaniline region of the *phox2b* protein (*Phox2b*^{27ala/+}), which is a common mutation in CCHS, failed to express glutamatergic *Phox2b*-positive neurons in the RTN and parafacial region, died shortly after birth and were insensitive to CO₂ (Dubreuil et al., 2008). The phenotype of these *Phox2b*^{27ala/+} transgenic mice was quite similar to the respiratory phenotype of CCHS.

Phox2b-labeled cells in the RTN are chemosensitive and form a distinct neuronal subtype based on morphology and neurotransmitter profile (*Phox2b*-labeled cells are glutamatergic, and none is cholinergic or catecholaminergic) (Lazarenko et al., 2009). The *Phox2b*-labeled cells within the RTN could be divided into two types, Type I and Type II, based on responses to hypercapnia. Type I cells had a lower baseline firing rates, and the maximal number of cells was active at pH 7.3. Type II cells fired more rapidly under normocapnic conditions and the maximal number of cells was active at pH 7.5. The cells had approximately the same range of firing frequencies, but the higher baseline rate in Type II cells at normocapnic physiological pH values enabled them to continue firing under more alkaline conditions.

The intrinsic chemosensitivity of neurons in the RTN is conferred, in part, by inhibition of a potassium leak current that shows little rectification and is not dependent on the calcium concentration. The leak conductance has the current-voltage characteristics of a TASK or TASKlike channel, but lacks sensitivity to halothane (which increases the open probability of the channels and hyperpolarizes neurons containing halothane-sensitive TASK channels) (Mulkey et al., 2004; Mulkey et al., 2007b). Transgenic mice lacking TASK1 or TASK3 or TASK1 and TASK3 channels responded normally to hypercapnia when studied as adults, and the chemosensory responses of individual RTN neurons were also normal in the TASK1 and TASK3 knockout mice (Mulkey et al., 2007b). Therefore, the pH sensitivity of RTN seems to originate from a TASK-like channel that is neither the TASK1 nor TASK3.

Recently, a TASK2 knockout mouse was created (Gestreau et al., 2010). TASK2 channels are not widely expressed in the CNS, but they are expressed in RTN neurons. The ventilatory response to CO₂ in the TASK2 knockout mice was shifted to the left so that the animals were more responsive to low levels of CO₂ (1–2%), but the ventilatory response to CO₂ reached a plateau at relatively low levels of inspired CO₂ (~5% rather than 6–7% in

wild-type mice). Overall, the slope of the ventilatory response to CO₂ was reduced slightly compared to wild type animals, but the change in CO₂ sensitivity was small. In addition, the decline in hypoxic ventilatory responses (the second element of the biphasic response to hypoxia that occurs after more prolonged exposure to hypoxia especially in newborn animals and small mammals) did not occur in the mutant mice. The interpretation of these studies is further complicated by additional phenotypic aspects of the TASK2 mutants; they develop renal tubular acidosis. Gestreau et al. (2010) mimicked the effect of this acidosis by adding ammonium chloride to the drinking water. Treatment with ammonium chloride did not replicate the respiratory effects of the TASK2 deletion, indicating that the TASK2 knockout phenotype did not originate simply from the metabolic acidosis.

These results are phenomenological at present: the TASK2 mutation caused a respiratory phenotype, but we do not have any information about the actual pH-sensitivity of the RTN neurons in the TASK2 mutant animals. The TASK2 mutant animals behaved as if some new tonic excitatory input were present so that the ventilatory response to CO₂ was augmented at low stimulus intensities, but the ventilatory response to elevated CO₂ may have been slightly reduced. In addition, the central inhibitory effects of hypoxia were antagonized. The actual electrophysiological response of RTN neurons to hypercapnia is, of course, vital to interpret the responses of the TASK2 knockout animals. In the absence of such data, one interpretation of the ventilatory activity of the TASK2 mutants is that TASK2 has nothing to do with the pH-sensitivity of RTN neurons. The animals had an intact ventilatory response to inhaled CO₂ despite lacking TASK2 channels (the changes in ventilatory sensitivity to CO₂ were subtle in the TASK2 mutants). Second, the TASK2 channel is unlikely to be the same pH-sensitive TASK-like channel studied previously in the RTN (Mulkey 2007), since TASK2 is sensitive to halothane. The low threshold of responsiveness in the TASK2 knockout mice may represent the loss of the constant hyperpolarizing TASK2 leak conductance in RTN cells – the resting membrane potential of RTN neurons in the TASK2 knockout mice should be less hyperpolarized and closer to the threshold for action potential generation and, therefore, hyper-responsive to any stimulus, all other things being equal (which, of course, they never are in knockout animals). The TASK2 channel may modulate respiratory activity, but it is not the sole chemosensory ion channel in these animals (if it contributes to CO₂ sensitivity at all) since these animals had a definite ventilatory response to hypercapnia (Gestreau et al., 2010).

In the same way, the maintenance of a vigorous response to hypoxia may reflect the persistent activity, in the absence of the hyperpolarizing TASK2 leak conductance, of activity of the RTN neurons, which may oppose central hypoxic depression. If this is true, it suggests that inhibition of the RTN, acting through TASK2 or some other inhibitory process, is an important part of the inhibitory phase of the biphasic response to hypoxia.

Some pre-inspiratory neurons in area C4, which encompasses the parafacial nucleus and may overlap the caudal elements of the RTN (the RTN has no strict, easily identified anatomical boundaries), are sensitive to hypercapnia (Kawai et al., 2006). Under conditions of synaptic blockade (high Mg⁺⁺/low Ca⁺⁺), these neurons generated increased numbers of action potentials in responses to acidotic stimuli. These pre-inspiratory neurons are, therefore, intrinsically chemosensitive (Leiter, 2009). In the presence of tetrodotoxin, hypercapnia depolarized the membrane potential and increased input resistance, from which we may infer that hypercapnia reduced the opening of ion channels conducting an outward current. Barium, but not cadmium treatment inhibited the depolarization of the membrane potential seen during hypercapnia in the presence of tetrodotoxin treatment. Barium inhibits a variety of potassium channels, and the response to barium is consistent with the changes in input resistance observed and consistent with the presence of a pH-sensitive potassium channel that mediates the hypercapnic depolarization of these neurons. However, the

response to barium differed depending on the nature of the acidosis. Membrane depolarization by hypercapnia (both intra- and extracellular pH change) was blocked by barium, from which we infer that a potassium channels was involved. On the other hand, neither barium nor cadmium prevented membrane depolarization during metabolic acidosis (a greater fall in extracellular than intracellular pH). TASK channels are inhibited by extracellular acidosis, but insensitive to barium. Perhaps TASK2 or the TASK-like channel identified by Mulkey et al. (2007b) or some other barium-insensitive outward conductance was inhibited by metabolic acidosis. In any event, the divergent responses to respiratory and metabolic acidosis imply that, as in other chemosensory nuclei, multiple channels are involved in neuronal responses to hypercapnia in the RTN.

5.4 Serotonergic Medullary Neurons

Brainstem serotonergic neurons form a diffuse neuronal network that is usually broken into a rostral group and a caudal group, which is comprised of the raphé magnus, raphé pallidus, and raphé obscurus nuclei in the midline of the caudal medulla, as well as a few serotonergic neurons located in the caudal ventrolateral medulla. A small number of cells in the ventromedial medulla and a smaller number of parapyramidal serotonergic neurons altered their firing rate in response to CO₂ when studied in brain slices (Richerson, 1995), although the majority of cells in these areas were unresponsive to hypercapnia. Neurons activated by hypercapnia were multipolar neurons with large somata and expressed tryptophan hydroxylase (indicating that they were serotonergic). The ionic mechanism underlying the intrinsic chemosensitivity of medullary raphé neurons is not well characterized. In the case of the serotonergic neurons activated by hypercapnia, it appears that the depolarization is associated with inhibition of an A-type current. There is also a robust expression of TASK1 and TASK3 channels in serotonergic raphé neurons (Washburn et al., 2002). Slice recordings in rats as young as P1 revealed that there was a TASKlike potassium conductance that was inhibited by halothane and pH with a pKa of around 7.0. Single and double deletion of TASK1 and TASK3 made the serotonergic raphé neurons completely insensitive to CO₂ in neonatal animals (Mulkey et al., 2007a), and this is in striking contrast to the effect of TASK1 and TASK3 double deletion on the RTN. Deletion of TASK1 and/or TASK3 had no effect on the ventilatory response to CO₂ in intact animals and no effect on the pH-sensitivity of neurons in the RTN. In summary, deletion of TASK1 and TASK3 ablates the chemosensory response of serotonergic neurons in the caudal raphé without affecting the whole animal ventilatory response to CO₂, but there may be additional pH-sensitive channels in non-serotonergic neurons in the caudal raphé that remain to be characterized.

5.5 Open questions

A major unanswered issue in the foregoing studies is that TASK1, TASK2 and TASK3 are all leak conductances and the response to knocking out these channels should, on the face of it, be similar. TASK1 and TASK3 deletion in the caudal raphé blocked the neuronal response to hypercapnia in neonatal animals, but had no effect on CO₂ sensitivity in RTN neurons even though a TASK-like channel is expressed in RTN neurons. Most interesting of all, the TASK1 and TASK3 mutants had normal ventilatory responses to CO₂ despite having reduced neuronal responses to CO₂ in the caudal raphé. TASK2 mutation in the RTN, on the other hand, slightly enhanced the ventilatory response to low levels of CO₂ in the whole animal. The TASK1, TASK2 and TASK3 channels have similar current-voltage relationships and are sensitive to extracellular pH, and all seem to participate in setting the resting membrane potential. One might expect them to have identical effects on neuronal and whole animal chemosensory response in the RTN and in the caudal raphé – but they don't. The question is: Why wasn't the response to TASK1/3 mutation in the caudal raphé like the TASK2 response in the RTN? If TASK channels contribute to the resting membrane

potential and are pH sensitive, then the simplest prediction is that loss of the hyperpolarizing TASK channel should increase baseline neuronal activity, but abrogate pH sensitivity. This prediction is actually borne out in studies of carotid body sensitivity to hypercapnia in TASK1 or TASK3 knockout animals (Trapp et al., 2008). Baseline carotid nerve activity recorded *in vitro* was increased in TASK3 knockout animals, and baseline carotid nerve activity was increased, and hypercapnic sensitivity was reduced in the TASK1 knockout animals. It is possible to interpret the TASK2 knockout animal responses in a similar way. The loss of the hyperpolarizing TASK2 channel may have depolarized and enhanced the activity of RTN neurons so that excitatory responses to low levels of CO₂ and to hypoxia were enhanced. The changes in the overall ventilatory response to hypercapnia were subtle, and for the time being it is probably simplest to accept the null hypothesis - no change in hypercapnic sensitivity. This interpretation means that TASK2 is a leak conductance in RTN neurons and contributes to baseline activity of RTN neurons, but the pH sensitivity of TASK2 does not actually contribute to the ventilatory response to hypercapnia. The TASK2 knockout studies are difficult if not impossible to interpret meaningfully without electrophysiological studies of the neuronal responses to CO₂. But if loss of TASK2 activity actually blocked acid-sensitivity in RTN neurons, then the interpretation of the TASK2 results is different. An intact ventilatory response in awake TASK2 mutant animals makes it apparent that the RTN is not a dominant site of chemosensory function – the other sites within the pons and medulla must have compensated for this hypothetical loss of pH-sensitivity in the RTN.

Turning now to the TASK1 and TASK3 effects in the raphé: Why wasn't the effect of knocking out TASK1 and TASK3 channels in the caudal raphé similar to the effect of knocking out TASK1 or TASK3 on the carotid body activity? There is no apparent change in baseline activity of the serotonergic neurons in the TASK1/3 knockout animals compared to the wild-type animals. The firing rate of raphé neurons in the TASK1/3 knockout animals was actually reduced compared to wild-type animals even though pH-sensitivity of these neurons was absent in the knockout animals (Mulkey et al., 2007b). Although CO₂ sensitivity was lost, the ventilatory response to CO₂ was normal. This despite the fact that injections of acetazolamide or hypercapnic saline in the caudal raphé stimulated ventilation (Bernard et al., 1996; Nattie and Li, 2001). There are non-serotonergic neurons in the caudal raphé, and they may play a more important role in the ventilatory response to CO₂ than has been appreciated. Alternatively, it may be that caudal raphé neurons play only a modulatory role in chemosensory function, and there is evidence that caudal raphé neurons affect chemosensory mechanisms through an RTN-dependent mechanism (Dias et al., 2008). The absence of pH-sensitivity of caudal raphé neurons in the TASK1 and TASK3 knockout mice may not change the ventilatory response to CO₂ much in animals in which RTN function is intact (and in which the RTN may have compensated in these knockout animals over the course of development). The hypothetical nature of this analysis makes it clear how vital further detailed electrophysiological studies of hypercapnic responses of individual neurons in normal and mutant animals are to allow interpretation of the divergent behavioral responses to knockout of TASK channels and other pH-sensitive channels.

6. Summary

The overall chemosensitivity of the brainstem has its foundations at the molecular level in pH-sensitive neuronal processes, but the flexibility, sensitivity and robustness of the hypercapnic ventilatory response are best explained by emergent properties that arise from the network interactions among chemosensory nuclei and other respiratory-related nuclei. At its core, chemosensitivity is effected by a reversible interaction between titratable groups, usually the imidazole group of histidine, in key regions of membrane ion channels and protons. The proton-protein interactions and associated changes in protein conformation

transform the sensory event into meaningful neuronal activity. Each channel's conductance or its open/closed probability comes to vary as a function of pH, and to either hyperpolarize or depolarize the cell, depending on the charge and direction of flow of ions. The location of the titratable groups on the channel determines whether it is the intracellular or the extracellular pH that is being sensed. The degree of intrinsic chemosensitivity of a neuron at a given pH is determined by the ratio of the current that is being carried by pH-sensitive ion channels to the net current and also by the slope of the pH-current relationship of the chemosensitive conductances. However, this linear approximation holds only for small excursions from the set-point pH, as most pH-sensitive currents have a sigmoidal dependence on pH and are maximally sensitive around their pKa, but the response saturates at the two extremes of pH. Thus, the range of the neuron's chemosensitivity can be broadened by having multiple chemosensitive conductances whose pKa's are slightly offset from each other.

We began this review by pointing out how the emphasis on chemosensory mechanisms has changed since 1982. However, we can in no way reject the work of Loeschke or any of the other investigators interested in synaptic mechanisms of central chemosensitivity. There has been an emphasis on ionic mechanisms in recent work, but the recent findings have to be integrated into and reconciled with previous work – no one has disproved Loeschke's hypotheses. Any new theory of chemosensitivity must incorporate the ideas of the past as well as more recent discoveries. Loeschke's work focused in essence on a synaptic process, acetylcholine transmission, which was enhanced by low pH. Acidosis mimicked the effect of inhibiting acetylcholine esterase. It is not hard to imagine that the ionic processes that we reviewed, which act at the level of the cell membrane on voltage-gated or voltage-dependent processes, may be integrated with a variety of pH-dependent synaptic events, which work at the level of the membrane on ligand-gated processes. pH-dependent modulation of synaptic events has not received much attention recently, but such events may still prove to be important elements in the central chemosensory process.

The process of chemosensitivity involves similar integration of multiple mechanisms at the network level. Individual cells have a limited range of firing frequencies, however, with subpopulations of neurons each tuned to their own center frequency, the population as a whole becomes sensitive to a wide pH range. The assignment of chemoreception to a large neuronal population (Erlichman et al., 2009) rather than an isolated cluster of cells renders the system more robust and flexible, integrating it within a larger network which includes respiratory rhythm generators, cardiovascular feedback mechanisms, sensory inputs, and circuits controlling sleep state. It also makes it a difficult system to study, since the experimenter's task of holding all variables constant except the one being measured is particularly hard to accomplish in a distributed system. The task is made all the more difficult in that the actual stimulus for sensing CO₂, a volatile acidic byproduct of aerobic metabolism, is relegated to the ubiquitous hydrogen ion, whose concentration is not uniform throughout the cell or brainstem, but varies by a factor of 10–100 and is dependent on metabolic activity, local buffering power, and the presence of heterogeneously distributed pH regulatory mechanisms. The range of pH values and the importance of pH regulation of the microenvironment around the chemosensory elements, the ion channels, has probably been underappreciated (Arita et al., 1989; Erlichman et al., 2004; Putnam, 2010).

A comparative analysis of chemosensory systems in invertebrates and vertebrates reveals that different animals use similar strategies in sensing CO₂ when controlling respiration. Respiratory chemoreceptors seem to rely largely on pH-dependent inhibition of potassium channels, but the channels do not appear to be uniquely adapted to detect pH. The 'chemosensory' potassium channels identified thus far are widely distributed, common potassium channels. The pH-sensitivity is a common feature of the channels whether they

are in chemosensory neurons or not. Thus, the pattern of synaptic connectivity and the mix of potassium channels expressed seem to determine whether a neuron is chemosensory or not, rather than any special adaptation of a channel for pH-sensitivity. Moreover, there are often multiple pH-sensitive channels in each chemosensory neuron. Creating a robust chemosensory neuron seems to require the simultaneous, coordinated activity of multiple pH-sensitive channels within single neurons (Chernov et al., 2007). The mechanisms of chemosensitivity are broadly similar among a diversity of air breathing animals. However, the similarities are often a result of convergence of analogous structures, each animal creating its sensor from the molecular building blocks it has at hand such that these elements can be integrated within the existing organism and confer maximal evolutionary advantage for survival within the animal's specific environmental niche.

Bibliography

- Amiel J, Laudier B, Attié-Bitach T, Trang H, de Pontual L, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, et al. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat. Genet.* 2003; 33:440–442. [PubMed: 12665862]
- Arita H, Ichikawa K, Kuwana S, Kogo N. Possible locations of pH-dependent central chemoreceptors: intramedullary regions with acidic shift of extracellular fluid pH during hypercapnia. *Brain Res.* 1989; 485:285–293. [PubMed: 2720414]
- Bernard DG, Li A, Nattie EE. Evidence for central chemoreception in the midline raphe. *J. Appl. Physiol.* 1996; 80:108–115. [PubMed: 8847290]
- Chernov MM, Daubenspeck JA, Denton JS, Pfeiffer JR, Putnam RW, Leiter JC. A computational analysis of central CO₂ chemosensitivity in *Helix aspersa*. *Am. J. Physiol.* 2007; 292:C278–C291.
- Coates EL, Li A, Nattie EE. Widespread sites of brain stem ventilatory chemoreceptors. *J. Appl. Physiol.* 1993; 75:5–14. [PubMed: 8376301]
- Coulter KL, Pèrier F, Radeke CM, Vandenberg CA. Identification and molecular localization of pH-sensing domain for the inward rectifier potassium channel HIR. *Neuron.* 1995; 15:1157–1168. [PubMed: 7576658]
- Dahr E. Studien über die Respiration der Landpulmonaten. *Acta Univ. Lundensis.* 1924; 20:1–19.
- Dean JB. Electrophysiological and morphology of CO₂-sensitive neurons in the dorsal vagal complex studied in vitro. *Soc. Neurosci.* 1990; 16:1235. (Abs.).
- Dean JB, Lawing WL, Millhorn DE. CO₂ decreases membrane conductance and depolarizes the nucleus tractus solitarii. *Exp. Brain Res.* 1989; 76:656–661. [PubMed: 2507342]
- Denton JS, McCann FV, Leiter JC. CO₂ chemosensitivity in *Helix aspersa*: Three K⁺ currents mediate pH-sensitive neuronal activity. *Am. J. Physiol.* 2007; 292:C292–C304.
- Dias MB, Li A, Nattie EE. Focal CO₂ dialysis in raphe obscurus does not stimulate ventilation but enhances the response to focal CO₂ dialysis in the retrotrapezoid nucleus. *J. Appl. Physiol.* 2008; 105:83–90. [PubMed: 18450988]
- Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet J-F, Goridis C. A human mutation in Phox2b causes lack of CO₂ chemosensitivity, fatal central apnea, and specific loss of parafacial neurons. *Proc. Natl. Acad. Sci.* 2008; 105:1067–1072. [PubMed: 18198276]
- Erlichman JS, Boyer AC, Reagan P, Putnam RW, Ritucci NA, Leiter JC. Chemosensory responses to CO₂ in multiple brainstem nuclei determined using a voltage-sensitive dye in brain slices from rats. *J. Neurophysiol.* 2009; 102:1577–1590.
- Erlichman JS, Cook A, Schwab MC, Budd TW, Leiter JC. Heterogeneous patterns of pH regulation in glial cells in the dorsal and ventral medulla. *Am. J. Physiol.* 2004; 286:R289–R302.
- Erlichman JS, Leiter JC. CO₂ chemoreception in the pulmonate snail, *Helix aspersa*. *Respir. Physiol.* 1993; 93:347–363. [PubMed: 8305038]
- Erlichman JS, Leiter JC. Central chemoreceptor stimulus in the terrestrial, pulmonate snail, *Helix aspersa*. *Respir. Physiol.* 1994; 95:209–226. [PubMed: 8191042]

- Erichman JS, Leiter JC. Identification of CO₂ chemoreceptors in *Helix pomatia*. *Am. Zool.* 1997; 37:54–64.
- Erichman JS, Leiter JC. Glial modulation of the extracellular milieu as a factor in central CO₂ chemosensitivity and respiratory control. *J. Appl. Physiol.* 2010 doi:10.1152/japplphysiol.01321.2009.
- Feldman JL, Mitchell C, Nattie EE. Breathing: Rhythmicity, plasticity, chemosensitivity. *Annu. Rev. Neurosci.* 2003; 26:239–266. [PubMed: 12598679]
- Filosa JA, Dean JB, Putnam RW. Role of intracellular and extracellular pH in the chemosensitive response of rat locus coeruleus neurones. *J. Physiol. (Lond).* 2002; 541.2:493–509. [PubMed: 12042354]
- Filosa JA, Putnam RW. Multiple targets of chemosensitive signaling in locus coeruleus neurons: role of K⁺ and Ca²⁺ channels. *Am. J. Physiol.* 2003; 284:C145–C155.
- Gallego J, Dager S. PHOX2B mutations and ventilatory control. *Respir. Physiol. Neurobiol.* 2008; 164:49–54. [PubMed: 18675942]
- Gesell R, Hansen ET. Eserine, acetylcholine and nervous integration. *Am. J. Physiol.* 1943; 139:371–385.
- Gestreau C, Heitzman D, Thomas JH, Dubreil V, Bandulik S, Reichold M, Bendahhopu S, Pierson P, Sterner C, Peyronnet-Roux J, et al. Task2 potassium channels set central respiratory CO₂ and O₂ sensitivity. *Proc. Natl. Acad. Sci.* 2010; 107:2325–2330. [PubMed: 20133877]
- Goldstein JJ, Mok JM, Simon C, Leiter JC. Intracellular pH regulation in neurons from chemosensitive and non-chemosensitive regions of *Helix aspersa*. *Am. J. Physiol.* 2000; 279:R414–R423.
- Jones WD, Cayirlioglu P, Kadow IG, Vossall LB. Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature.* 2007; 445:86–90. [PubMed: 17167414]
- Kawai A, Onimaru H, Homma I. Mechanisms of CO₂/H⁺ chemoreception by respiratory rhythm generator neurons in the medulla from newborn rats *in vitro*. *J. Physiol.* 2006:525–537. 572.2. [PubMed: 16469786]
- Kwon JY, Dahanukar A, Weiss LA, Carlson JR. The molecular basis of CO₂ reception in *Drosophila*. *Proc. Natl. Acad. Sci.* 2007; 104:3574–3578. [PubMed: 17360684]
- Lazarenko RM, Milner TA, Depuy SD, Stornetta RL, West GH, Kievits JA, Bayliss DA, Guyenet PG. Acid sensitivity and ultrastructure of the retrotrapezoid nucleus in Phox2b-EGFP transgenic mice. *J. Comp. Neurol.* 2009; 517:69–86. [PubMed: 19711410]
- Leiter JC. Intrinsic chemosensitivity: How is it measured; what does it mean; and how does it help us understand the ventilatory response to CO₂? *Respir. Physiol. Neurobiol.* 2009; 166:13–15. [PubMed: 19444986]
- Loeschcke HH. Central chemosensitivity and the reaction theory. *J. Physiol. (Lond).* 1982; 332:1–24. [PubMed: 6818338]
- Mulkey DK, Rosin DL, West GB, Takakura AC, Moreira TS, Bayliss DA, Guyenet PG. Serotonergic neurons activate chemosensitive retrotrapezoid nucleus neurons by a pH-independent mechanism. *J. Neurosci.* 2007a; 27 14218–14138.
- Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA, Guyenet PG. Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat. Neurosci.* 2004; 7:1360–1369. [PubMed: 15558061]
- Mulkey DK, Talley EM, Stornetta RL, Siegel AR, West GH, Chen XH, Sen N, Mistry AM, Guyenet PG, Bayliss DA. TASK channels determine pH sensitivity in select neurons but do not contribute to central respiratory chemosensitivity. *J. Neurosci.* 2007b; 27:14049–14058. [PubMed: 18094244]
- Nattie EE, Li A. CO₂ dialysis in the medullary raphe of the rat increases ventilation in sleep. *J. Appl. Physiol.* 2001; 90:1247–1257. [PubMed: 11247921]
- Niemeyer MI, González-Nilo FD, Zúñiga L, González W, Cid LP, Sepúlveda FV. Neutralization of a single arginine residue gates open a two-pore domain, alkali-activated K⁺ channel. *Proc. Natl. Acad. Sci.* 2007; 104:666–671. [PubMed: 17197424]
- Onimaru H, Ikeda K, Kawakami K. CO₂-sensitive preinspiratory neurons of the parafacial respiratory group express Phox2b in the neonatal rat. *J. Neurosci.* 2008; 28:12845–12850. [PubMed: 19036978]

- Pattyn A, Hirsch M, Goridis C, Brunet JF. Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. *Development*. 2000; 127:1349–1358. [PubMed: 10704382]
- Putnam RW. Role of pH in cellular CO₂ chemosensitivity. *J. Appl. Physiol.* 2010 doi:10.1152/japplphysiol.01169.2009.
- Rahn H, Reeves RB, Howell BJ. Hydrogen ion regulation, temperature, and evolution. *Am. Rev. Respir. Dis.* 1975; 112:165–172. [PubMed: 239616]
- Reeves RB. An imidazole alphastat hypothesis for vertebrate acid-base regulation: tissue carbon dioxide content and body temperature in bullfrogs. *Respir. Physiol.* 1972; 14:219–236. [PubMed: 4537783]
- Richerson GB. Response to CO₂ of neurons in the rostral ventral medulla *in vitro*. *J. Neurophysiology*. 1995; 73:933–944.
- Ritucci NA, Chambers-Kersh L, Dean JB, Putnam RW. Intracellular pH regulation in neurons from chemosensitive and nonchemosensitive areas of the medulla. *Am. J. Physiol.* 1998; 275:R1152–R1163. [PubMed: 9756546]
- Ritucci NA, Dean JB, Putnam RW. Intracellular pH response to hypercapnia in neurons from chemosensitive areas of the medulla. *Am. J. Physiol.* 1997; 273:R433–R441. [PubMed: 9249582]
- Somero GN. Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* 1986; 251:R197–R213. [PubMed: 3017133]
- Stornetta RL, Moreira TS, Takakura AC, Kang BJ, Chang DA, West GH, Brunet JF, Mulkey DK, Bayliss DA, Guyenet PG. Expression of Phox2b by brainstem neurons involved in chemosensory integration in the adult rat. *J. Neurosci.* 2006; 26:10305–10314. [PubMed: 17021186]
- Thom C, Guerenstein PG, Mechaber WL, Hildebrand JG. Floral CO₂ reveals flower profitability. *J. Chem. Ecol.* 2004; 30:1285–1288. [PubMed: 15303329]
- Trapp S, Aller MI, Wisden W, Gourine AV. A role for TASK-1 (KCNK3) channels in the chemosensory control of breathing. *J. Neurosci.* 2008; 28:8844–8850. [PubMed: 18753386]
- Wang W, Bradley SR, Richerson GB. Quantification of the response of rat medullary raphe neurones to independent changes in pH_o and P_{CO2}. *J. Physiol. (Lond)*. 2002; 540.3:951–970. [PubMed: 11986382]
- Washburn CP, Sirois JE, Talley EM, Guyenet P, Bayliss DA. Serotonergic raphe neurons express TASK channel transcripts and a TASK-like pH- and halothane-sensitive K⁺ conductance. *J. Neurosci.* 2002; 22:1256–1265. [PubMed: 11850453]