Central Chemosensitivity in Mammals

L.K. Hartzler and R.W. Putnam

Abstract More than a century has passed since the beginning of direct experimentation on control of ventilation, and the ensuing years have brought considerable insight into the mechanisms of this control. Much of what we know about cellular chemosensitivity in mammals comes from a limited number of species; yet, given the diversity of circumstances in which mammals exist, their potential has been greatly underused. Here we review some of the environmental situations for plasticity of mammalian central chemosensitivity and function of chemosensors. "Normal" breathing patterns change during sleep, hibernation, and exercise, and central chemosensitivity must be altered during acclimation or adaptation to altitude, burrowing, or disease states. Where central chemosensitive cells are located, and what qualifies a cell as chemosensitive, is currently debated. The chemosensitivity of these cells changes over development, and the signaling mechanisms of these cells vary between chemosensitive regions, probably accounting for plasticity in response to environmental perturbations.

1 Introduction

More than a century has passed since the beginning of direct experimentation on the control of ventilation, and the ensuing years have brought considerable insight into the mechanisms of this control. In this chapter we highlight a few of these ideas and present directions in which central chemosensitivity in mammals are going. Haldane's experiments at the turn of the century demonstrated that, in humans, the ventilatory control center is exquisitely sensitive to changes in $[CO_2]$ while a relatively larger change in $[O_2]$ is required to get an equivalent increase in ventilation

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(Haldane and Priestley 1905). This led to investigations into peripheral and central chemosensitivity (where and how blood gases are sensed) and how breathing is altered accordingly. Here we focus on central chemosensitivity. While central chemosensitivity has long been associated with terrestrial animals, its presence in aquatic air-breathers suggests that it is an ancient ventilatory control mechanism (Remmers et al. 2001; Wilson et al. 2000).

There are many similarities in control of breathing between all of the vertebrate classes, but mammals have a few unique characteristics. During normal metabolic states, most mammals have a regular breathing pattern (no periods of apnea) at rest, alter their ventilation with changes in $[CO_2]$ and not $[O_2]$ (until oxygen levels are quite depressed), and keep blood gas concentrations within a narrow range compared with ectothermic vertebrates (for further information, see Chaps. 3 and 5). A number of conditions observed in mammals result in altered breathing patterns, possibly due in part to changes in central chemosensitivity. For example: some groups of mammals undergo frequent bouts of torpor, and, more extensively, periods of hibernation; fossorial life histories result in altered CO_2 sensitivity; some mammals dive for extended periods of time, accumulating CO_2, thus altering central CO_2 sensitivity; acclimatization or adaptation to high altitude leads to changes in blood pH and O_2 sensitivity; and, in humans at least, a number of disease states result in or from altered central chemosensitivity. Additionally, central control of breathing rapidly responds to changes in metabolic state (e.g., sleep vs wakefulness, onset of exercise, anesthesia). In the following sections of this chapter, we discuss how the organismal response of central chemosensitivity is altered during these situations, where and how the chemical signals are sensed, and some of the ways in which this information is being put to use. Due to space limitations and given our broad approach, not all of these topics can be explored in detail, so a number of excellent reviews will be recommended where appropriate.

2 Organismal Chemosensitive Responses to Environmental or Metabolic Stimuli

Being endothermic, most mammals maintain constant, high body temperatures, but, during periods of environmental stresses, some mammals accommodate by entering periods of torpor or, more extensively, hibernation. The molecular bases for entrance into hibernation and subsequent arousals are not yet known (Carey et al. 2003), but it is a regulated metabolic depression that protects hibernators from cell death (Boutilier 2001). During hibernation, the rate of ventilation slows and becomes episodic (Nicol and Andersen 2003), and is accompanied by a decrease in the respiratory exchange rate, possibly by a retention of carbon dioxide in body fluids (Malatesta et al. 2007). Even during euthermia, the low metabolism of monotremes is matched by lower and variable rates of ventilation (Nicol and Andersen 2003).

Mammals that undergo hypometabolic states (torpor) switch from eupnea to intermittent breathing (Malan 1982; Milsom 1992). Torpid bats switch between

these two breathing strategies to optimize ventilatory requirements to meet metabolic demand (Szewczak 1997). Intermittent breathing has the consequence of high fluctuations in arterial pH [up to 0.24 pH unit in *Eptesicus fuscus* (Szewczak and Jackson 1992b)] during the ventilatory cycle. Relatively large changes in pH and periods of apnea are characteristic of ectothermic vertebrates, so it appears that when mammals undergo periods of torpor, even transient periods, they express "ectothermic breathing patterns" rather than maintaining eupnea (Bickler 1984). During torpor, the cost of ventilation is about 5–10% of all metabolic costs, so optimizing the ventilatory strategy becomes important (Szewczak 1997). With such large fluctuations in arterial pH and Pco_2, it appears that chemosensitivity is decreased, yet *E. fuscus* (Szewczak and Jackson 1992a) and *Paragnathus longimembris* (Withers 1977) consistently respond to hypercapnia and hypoxia.

While there is variability between species in breathing pattern during hibernation, the same chemical signals contribute to changes in those patterns. A strong hypoxic ventilatory response and a blunted hypercapnic ventilatory response during euthermia suggest that arterial Po_2 predominates over arterial Pco_2 and pH as a ventilatory control mechanism in echidnas (Parer and Hodson 1974), ground squirrels (McArthur and Milsom 1991), and bats (Szewczak and Jackson 1992b). However, during hibernation this hypoxic ventilatory response is greatly reduced, and the hypercapnic ventilatory response is elevated (McArthur and Milsom 1991; Nicol and Andersen 2003; Szewczak and Jackson 1992b). While changes in arterial Po_2, Pco_2, and pH all contribute to changes in ventilation during entrance into hibernation in *Spermophilus spp.* (McArthur and Milsom 1991), arterial Pco_2 and pH are primarily responsible for changes in ventilation during hibernation in ground squirrels (McArthur and Milsom 1991) and hedgehogs (Tahti 1975; Tahti and Soivio 1975). Since many hibernators are also fossorial, they typically exhibit a reduced hypercapnic ventilatory response (Milsom et al. 1993). This reduced hypercapnic ventilatory response in burrowing mammals (Boggs et al. 1984) is a probable adaptation to the chronic hypercapnic (and hypoxic) environments of burrows (Milsom 1998). Ventilatory and acid–base adaptations vary widely among fossorial mammals (Birchard et al. 1984; Lechner 1976; Walker et al. 1985).

While hypothermia is normal in animals that regularly undergo periods of torpor, for those animals that cannot enter a torpid state, progressive hypothermia eventually results in death, as opposed to the long apneas typical during hibernation (Rosenhain and Penrod 1951). Neonatal animals retain a greater tolerance to hypothermia than do adults (Mortola 2001). This loss of ventilatory control during hypothermia appears to occur at the network level rather than within the chemosensitive neurons (Mellen et al. 2002) and has interesting implications for sleep-disordered breathing in humans. Even in hibernators, hypothermia affects neural connections (von der Ohe et al. 2007) by dissociating synaptic proteins from synapses. Only very recently have the unique characteristics of control of ventilation during hibernation begun to be examined at the neuronal level (Drew et al. 2007; Ruediger et al. 2007).

One characteristic shared by many hibernators is a fossorial lifestyle (Milsom 1992). This life history trait can lead to burrowing mammals being periodically

exposed to high CO_2 environments without a depressed metabolic state. These mammals retreat to their burrows to escape predation, raise their young, or minimize fluctuations in environmental temperature. While some burrows are well-ventilated (Vogel et al. 1973), others have relatively high CO_2 levels. As inspired CO_2 triggers changes in ventilation, the properties of chemosensitive neurons in fossorial mammals should give us valuable insight into adaptation to high CO_2 in neuronal areas (Ar et al. 1977; Arieli and Ar 1979; Boggs et al. 1998; Chapman and Bennett 1975; Darden 1972). Under fossorial conditions they are no longer as CO_2-sensitive as non-fossorial mammals, suggesting a change in either cellular chemosensitive signaling pathways and/or chemosensitive neuronal networks.

Increases in CO_2 production during exercise are exquisitely matched by concomitant increases in ventilation (see Chap. 20 for extensive discussion of exercise). There are examples, however, of high metabolic demand without the usual capacity for normal gas exchange to accommodate this high demand, such as the extended dive times in several marine mammal species (Kohin et al. 1999; Parkos and Wahrenbrock 1987). No external gas exchange during dives is a source of periodically elevated arterial Pco_2 levels. Weddell seals, elephant seals, and other amphibious species actively forage for long periods of time: a metabolically expensive activity resulting in relatively large swings in blood oxygen and carbon dioxide levels (for example, venous Po_2 as low as 2 Torr has been reported in elephant seals (Meir et al. 2008)). Deep-ocean dwelling cetaceans must experience similar swings in blood gases as well as the additional factor of experiencing hyperbaric pressures. Even in humans, extreme breath-hold divers show a blunted hypercapnic ventilatory response (Eynan et al. 2005; Ferretti 2001; Masuda et al. 1982). Interestingly, some animals' ventilatory mechanics are coordinated with their locomotion (Boggs 2002), and the input from chemical stimulation to breathe is not strong enough to alter ventilation during locomotion (Gillespie et al. 1991). It is clear that there is a complex interplay among metabolism, temperature regulation, and environmental conditions which alters ventilatory control, and these interplays are poorly understood.

3 Altered Chemosensitivity in Disease

Humans are one of the mammalian species that keep blood gases within a very narrow range; however, there are a number of circumstances under which this is not the case. Diseases such as chronic obstructive pulmonary disease, sleep apnea, idiopathic hyperventilation, and chronic heart failure all result in blood gas values (especially CO_2) at a new set point (Dunroy et al. 2003; Jack et al. 2004; Kara et al. 2003). Additionally, individuals who are mechanically ventilated after respiratory failure are often chronically hypercapnic (as a result of low tidal volumes to prevent over-inflation of the lung), which may increase time to wean from a ventilator (Georgopoulos 2006; Laffey and Kavanagh 2006). While humans normally do not experience periods of torpor, artificially induced hypothermia during open thoracic surgery is becoming a common practice; however, whether to keep arterial blood

at pH 7.4 and allow Pco_2 to rise, or to maintain arterial Pco_2 at 40 Torr and allow pH to rise, has not been determined (Kofstad 1996). Further complications during surgery include the effects of anesthetic agents on ventilatory control (Dahan and Teppema 2003). Interestingly, a number of respiratory control disorders appear to be related to sleep states, so this may be a critical time during which the regions of chemosensitive neurons associated with the sleep state swamp the input from other regions (supporting the distributed chemosensitive regions model discussed below). Once again, understanding the relationships between ventilatory control during sleep states, changes in metabolic state (such as torpor), and temperature are important.

4 Development of the Organismal Chemosensitive Response

At birth the respiratory system of mammals must respond to very different ambient gases. Having developed in a relatively hypoxic environment in utero, neonates are essentially exposed to an initially hyperoxic environment. Because there is such a range of developmental stages at which birth occurs (e.g., highly altricial marsupials and precocial ungulates), there is great potential to examine the characteristics of ventilatory control centers and central chemosensitivity during the transition from the uterus (or egg in the case of monotremes) to ambient air (Bavis et al. 2006). The presence of fetal breathing movements in utero increases over the gestational period (Berger et al. 1986), and these movements also increase in response to hypercapnia and decrease in response to hypocapnia (Jansen et al. 1982; Van Weering et al. 1979), indicating fetal chemosensitivity to blood gas levels. The mechanisms by which irregular fetal breathing movements transition to well-regulated breathing at birth remain unknown (Mortola 2001). It is clear that neural control of ventilation is not as mature in pre-term infants as it is in those full-term (Darnall et al. 2006), and CO_2 chemosensitivity continues to change during the developmental period (Carroll 2003; Davis et al. 2006; Stunden et al. 2001).

5 Central Chemosensitivity

Much of what we know about cellular chemosensitivity in mammals comes from a limited number of species; yet, given the diversity of circumstances in which mammals exist, their potential has been greatly underused. Both peripheral (see Chap. 18) and central chemoreceptors are sensitive to changes in CO_2; with peripheral chemoreceptors removed, the ventilatory responses to CO_2 are reduced, indicating the influence of peripheral chemoreceptors on central integration of blood gas stimuli. As central CO_2 sensitivity returns after the removal of peripheral chemoreceptor input, it is clear that there is plasticity in the central chemoreceptors

themselves (Dahan et al. 2007). Where blood gases are sensed centrally is not clear, although there are a number of putative chemosensitive regions (Feldman et al. 2003; Hodges et al. 2004; Nattie and Li 2006, 2008); the contributions of numerous sites is currently under debate (Guyenet et al. 2008; Nattie and Li 2008). Criteria for areas of central chemosensitivity include an increase in ventilation during focal acidification, a change in ventilation with the inhibition or lesion of neurons, CO_2-responsive neurons in in vitro preparations, and/or c-fos expression after exposure to CO_2 (Putnam et al. 2004). Using a combination of these criteria, regions of putative central chemosensitivity have been identified within the ventral–lateral medullary surface (Akilesh et al. 1997; Biancardi et al. 2007; Guyenet et al. 2005; Leiter et al. 2003; Mulkey et al. 2004, 2007; Paterson et al. 2006; Ritucci et al. 2005b), locus coeruleus (A6 neurons) (Ballantyne et al. 2004; Biancardi et al. 2007; Filosa et al. 2002; Hartzler et al. 2008a; Li and Nattie 2006; Nichols et al. 2008; Ritucci et al. 1998), solitary tract nucleus (Dean et al. 1990a, 1997; Kline et al. 2002; Mulkey et al. 2003; Nichols et al. 2008; Ritucci et al. 1998), medullary raphé (Bernard et al. 1996; Bouyer et al. 2004; Bradley et al. 2002; Cao and Song 2006; Hodges et al. 2004; Paterson et al. 2006; Richerson et al. 2001), preBötzinger complex (Feldman et al. 2003; Neubauer and Sunderram 2004), and the fastigal nucleus (Martino et al. 2007).

Considerable progress has been made in the study of central chemosensitivity over the past 20 years and several recent reviews highlight various aspects of the subject (Feldman et al. 2003; Nattie and Li 2006; Putnam et al. 2004). Despite the progress, several significant questions remain which are major foci of research. We will highlight four questions. One important question is why there are so many brainstem sites that are chemosensitive and what might be the role of each of these areas in chemoreception. In addition, it is not clear what the developmental pattern of central chemoreception is in altricial species such as humans and rats, and whether there might be a "neonatal" form of chemoreception that is distinct from "adult" chemoreception. New data are also suggesting a re-interpretation of the role of central vs peripheral chemoreception in the ventilatory response to hypercapnia. Finally, the cellular mechanism(s) involved in transducing a change in CO_2/H^+ into altered neuronal firing rate are not fully elucidated.

6 Multiple Chemosensitive Regions in the Brainstem?

Currently, there are two main theories for the basis for central chemosensitivity in mammals: the distributed chemoreception theory and the specialized chemoreception theory (Guyenet et al. 2008; Nattie and Li 2008). For years, the sensing of elevated CO_2 was believed to reside in a few specialized areas in the ventral medulla (Loeschcke 1982; Mitchell et al. 1963). This view came to be challenged by two different approaches that suggested that CO_2 sensing is more distributed, residing in multiple brainstem sites. The first approach was studying individual chemosensitive neurons in a dorsal medullary site, the nucleus tractus solitarius (NTS) (Dean et al.

1989; Miles 1983). In these studies, between a third and two-thirds of the neurons within the NTS were found to respond reversibly, with an increased firing rate in response to acute exposures to hypercapnia. It was further shown that this neuronal response was intrinsic, because the increased firing rate in response to hypercapnia was still seen in synaptic block medium (Dean et al. 1990b). Significantly, the hypercapnia-activated response of NTS neurons occurred even in brainstem slices in which the ventral medulla had been removed, eliminating any possibility that the response resulted from activation of the ventral medulla (Dean et al. 1990b). These findings suggested that sites other than the ventral medullary surface could be involved in central chemosensitivity.

The other approach that supported the suggestion of multiple sites of central chemosensitivity was the use of focal acidosis in restricted brainstem sites within the intact animal. Coates et al. (1993) found that the output of the phrenic nerve, the major nerve supplying the diaphragm, increased when various brainstem sites, including the retrotrapezoid nucleus (RTN), the caudal NTS, and the locus coeruleus (LC), were exposed to focal acidosis. Since this initial work, additional sites have been reported that increase ventilation (or phrenic nerve activity) when focally acidified, including the medullary raphé (Bernard et al. 1996; Hodges et al. 2004; Nattie and Li 2001), the pre-Bötzinger complex (PBC) (Solomon et al. 2000) and the fastigial nucleus of the cerebellum (Xu et al. 2001). Interestingly, focal acidosis of each of these areas raised ventilation by only a fraction (usually 10–30%) of the increase in ventilation induced by the animal breathing hypercapnia, strongly indicating that no single area was sufficient to give a full central chemoreceptive response to increased inspired CO_2 (Nattie 2001). It is also noteworthy that most of the areas that result in increased ventilation when focally acidified contain neurons that are reversibly activated by elevated CO_2/H^+, including in the RTN (Mulkey et al. 2004; Ritucci et al. 2005b), the NTS (Dean et al. 1989, 1990a; Miles 1983), the LC (Filosa et al. 2002; Filosa and Putnam 2003; Oyamada et al. 1998; Ritucci et al. 2005a) and the medullary raphé (Richerson et al. 2001; Wang et al. 2001). These studies have led to the theory that the sensing of CO_2 by the central nervous system arises from numerous chemosensitive regions distributed throughout the brainstem, and has thus been termed the distributed chemoreception theory (Guyenet et al. 2008; Li et al. 2008; Nattie and Li 2006, 2008).

The distributed chemoreception theory has further been strengthened by studies of cell-specific lesions in various putative chemoreceptive regions. The concept of such studies is to eliminate specific cell types (e.g. serotonin- or catecholamine-expressing neurons) and determine the effect of this on central chemosensitivity. Many of these studies employed the approach of targeting neurons that produce specific neurotransmitters, so an added benefit is that insight has been gained into the neurotransmitters involved in central chemoreception as well. The major findings of such work have recently been briefly reviewed (Nattie and Li 2008). For instance, injection of ibotenic acid (a toxin for excitatory amino acids) into the RTN resulted in a loss of 35% of the neurons and a decrease of 39% in the CO_2/H^+-induced increase in ventilation (Akilesh et al. 1997), suggesting that glutamatergic RTN neurons are involved in central chemoreception. This is further indicated by

inhibiting RTN neurons with focal injection into the RTN of the GABA-agonist muscimol, which results in a 24% reduction of the response of ventilation to inspired CO_2 (Li et al. 2006). Another group of neurons that have been indicated in central chemoreception are serotonergic neurons of the medullary raphé. In mice in which serotonergic neurons have been knocked out the ventilatory response to inspired hypercapnia is decreased by 50% (Hodges et al. 2008). Using a different approach, medullary serotonergic neurons were killed by attaching the cytotoxic agent saporin to an antibody to the serotonin receptor. In such animals, ablation of medullary serotonergic neurons reduced the hypercapnic ventilatory response by about 17% in both awake and asleep rats (Nattie et al. 2004). Similar injections in the raphé magnus (which contains about 20% serotonergic neurons) reduced the hypercapnic ventilatory response by as much as 62% (Dias et al. 2007). Interestingly, lesions of serotonergic neurons from the caudal raphé had no direct effect on the hypercapnic ventilatory response but reduced the response of RTN neurons (Li et al. 2006), suggesting that serotonergic neurons from the rostral regions of the medulla are involved in central chemoreception, but those from the caudal regions serve to modulate the response of RTN neurons to hypercapnia (Nattie and Li 2008). Finally, catecholaminergic neurons from the pons also have been shown to play a significant role in central chemoreception. These neurons can be lesioned by complexing saporin to an antibody to an enzyme involved in catecholamine synthesis (dopamine-β-hydroxylase), which results in a reduction of the hypercapnic ventilatory response by 28% when injected into the cisterna magna (Li and Nattie 2006). The effect is even more dramatic when the lesion is focused on the LC, decreasing the hypercapnic ventilatory response by 64% (Biancardi et al. 2007).

Taken together, these ablation experiments show that no one area is completely responsible for the hypercapnic ventilatory response and support the idea that central chemoreception is a distributed phenomenon. This concept is further supported by the fact that many of these putative chemosensitive neurons contain receptors for substance P (NK1 receptors), including neurons from the LC (Chen et al. 2000), the RTN (Stornetta et al. 2006), and broadly throughout the ventral medulla (Nattie and Li 2002, 2006). Focal lesions of NK1-expressing neurons reduce the hypercapnic ventilatory response by at most 30% (Nattie and Li 2002, 2004), but broader lesions throughout the ventral medulla cause a much larger reduction in the hypercapnic ventilatory response, by as much as 65% (Nattie and Li 2006). Thus, there is considerable evidence supporting the hypothesis that central chemoreception arises from a distributed network of chemosensitive regions.

An interesting suggestion to arise from the distributed chemoreception theory is that chemoreception arose in a hierarchical fashion (Nattie 2001). Different sites involved in the control of ventilation may have arisen at various landmark stages in phylogeny. Such landmarks may include emergence from an aqueous environment to become air-breathing, the development of a high constant body temperature, the need for sleep, and the evolving of different stages of sleep. In such a hierarchical system, different chemoreceptive sites may not so much reflect duplication of an important physiological process as the accretion of new sites involved in a different aspect of ventilatory control. Such a hierarchical concept is entirely consistent

with the observation that chemoreception is clearly state-dependent, varying with states such as being awake, anesthetized or asleep. The response of chemosensitive neurons from various regions is also state-dependent. For instance, focal acidosis of the RTN increased the hypercapnic ventilatory response only in awake, but not sleeping, rats, and the effect was largely due to increased tidal volume (Nattie 2000). In contrast, focal acidosis in the medullary raphé increased ventilation only during sleep and the effect was entirely due to an increased respiratory frequency (Nattie 2000). Loss of catecholaminergic neurons reduced the hypercapnic ventilatory response during both wakefulness and sleep (Li and Nattie 2006). In fact, it is intuitively obvious that central chemoreception is state-dependent, given the number of disordered breathing states associated with sleep (e.g. congenital central hypoventilation syndrome, sleep apnea, sudden infant death syndrome) that are not apparent in the awake animal (Feldman et al. 2003). The state dependence of central chemoreception is most consistent with central chemoreception being a distributed property.

In contrast to the distributed chemoreception theory, it has very recently been argued that chemoreception for the purpose of controlling ventilation is largely (if not exclusively) the function of specialized neurons restricted to a specialized region, probably within the RTN. This theory has been termed the specialized chemoreceptor theory (Guyenet et al. 2008). It is noteworthy that this theory is similar to the original theory that chemoreception resides in the ventral medullary surface. In fact, the putative specialized chemoreceptive region within the RTN (Mulkey et al. 2004) markedly overlaps one of the originally proposed ventral chemoreceptive regions, the M region (Loeschcke 1982), although, while focusing on this region as *the* central chemoreceptor, no further comment is made about the other two classical chemosensitive regions on the ventral medulla. Evidence, as discussed above, suggests that RTN neurons play a role in central chemoreception. Further, glutamatergic neurons within a specialized region of the RTN have been shown to be highly and intrinsically responsive to elevated CO_2 (Mulkey et al. 2004). Also, in knock out mice lacking the transcription factor Phox2b, there is a marked deficit of RTN neurons and a complete loss of the ventilatory response to inspired CO_2 (Dubreuil et al. 2008). Finally, lesions of the medullary raphé and the RTN decrease the ventilatory response to inspired CO_2 by half (Li et al. 2006). Thus, there appears to be an important role for RTN neurons in central chemosensitivity.

Evidence for a significant role of RTN neurons in central chemoreception does not negate the likelihood that several brainstem regions contribute to central chemoreception. Many of the arguments against neurons from other regions playing a role in central chemoreception are arguments against being able to claim that any neuron that responds to acidification must be a central chemoreceptor (Guyenet et al. 2008). The claim is made that many neurons may not act the same in in vitro preparations as they do in vivo. While this is undoubtedly so, measurements of the responses of individual or populations of neurons in vivo must of necessity involve studies of anesthetized animals and are thus highly modified preparations in their own right. Further, the vast majority of studies on the chemosensitive response of individual neurons in reduced preparations are done studying neurons in areas that have already

been shown to be involved in central chemoreception based on focal acidosis or neuronal ablation experiments (Putnam et al. 2004). Guyenet et al. (2008) do not address this point in any substantive way other than to suggest that indwelling cannulae used for focal acidosis can alter the local circuit. However, this argument does not account for the lack of a response when control solutions are infused through the cannulae or when non-chemosensitive regions are being studied. Finally, while studies of the Phox2b knockout mice are interesting, there are Phox2b-containing neurons in many chemosensitive areas and, although the Phox2b neurons from these areas do not disappear as they do from the RTN, there are no electrophysiological studies of neurons from these regions to determine whether they are altered in knockout animals as well. Also, as pointed out by Nattie and Li (2008), there may be other neurons affected by Phox2b knockout, especially peripheral chemoreceptors, that account for some of the loss of chemoreception. In summary, while it is likely that RTN neurons play a role in some aspects of central chemoreception, it appears most reasonable to assume that central chemoreception arises from a distributed network of chemosensitive neurons within numerous regions of the brainstem (and possibly the cerebellum).

7 Development of Central Chemoreception

The development of physiological processes is often assumed to be an essentially linear process, progressing from a poorly functioning state at birth, through early development, until eventually reaching the adult state. An alternative view of development is that there are distinct physiological states, e.g., a neonatal state vs the adult state, and that physiological processes and their regulation are adapted for each state. Both of these views have been posited for the development of central chemoreception.

A linear development of chemosensitivity, especially within medullary raphé neurons, has been suggested (Richerson et al. 2001; Wang and Richerson 1999). Medullary raphé neurons in slices and especially in culture showed increased numbers of chemosensitive neurons and a larger response to high CO_2/H^+ with age (or days in culture) (Richerson et al. 2001; Wang and Richerson 1999), suggesting that central chemoreception increases with age in neonatal rats. A variation on this pattern has also been described, with the ventilatory response to inspired CO_2 remaining low from P0 to about P14, and then increasing rapidly after P15 to adult levels (Davis et al. 2006). The timing of this increase in the hypercapnic ventilatory response is interesting in that shortly before this time (around P11–P12) there are a number of transient changes in various neurotransmitter systems in neurons from many different medullary chemosensitive regions (Wong-Riley and Liu 2005). This pattern of development raises the possibility that there is a critical signal generated near the end of the second week of life in rats that causes a rapid increase in the hypercapnic ventilatory response.

A different pattern of development of chemosensitivity in neonatal rats has been suggested. Stunden et al. (2001) found that the hypercapnic ventilatory response to inspired CO_2 was fairly large shortly after birth (P0–P1) and decreased after that time to a point with virtually no ventilatory response to inspired CO_2 from about P6 to P10. After P10, the hypercapnic ventilatory response increased again to adult levels. This latter increase is similar in timing to that described by Davis et al. (2006) and shows general agreement that there is indeed an increase in the hypercapnic ventilatory response near the end of the second week of life in rats. The difference is in the large hypercapnic ventilatory response early in development which was seen by Stunden et al. (2001) but not by Davis et al. (2006). The latter authors attributed the difference to the need to express increased ventilation as a percentage change without normalizing for body weight, but this cannot account for the difference since Stunden et al. (2001) also express increased ventilation with hypercapnia as a percentage increase (Fig. 10 in Stunden et al. 2001). Further, this early increase in ventilation with inspired hypercapnia is due largely to an increase in respiratory frequency (unpublished observation). A similar response of hypercapnia-induced increase in respiratory frequency in young neonatal rats, but not older neonates, was also observed by Wickström et al. (2002). These authors concluded, like us, that the hypercapnic ventilatory response decreased during the first week of life in rats but increased again thereafter, the increase being largely due to increased tidal volume with hypercapnia.

This triphasic pattern of ventilatory response to hypercapnia (Putnam et al. 2005; Stunden et al. 2001), with a large response early in development, a loss of response near the end of the first week of life, and an increase again after about P10, has prompted us to hypothesize that in rats there are two forms of central chemoreception, a "neonatal" form and an "adult" form. Since rats are born highly altricial, without the ability for locomotion or temperature regulation, we would presume that neonatal chemoreception would be relatively simple, without the need for subtle responses such as exercise hyperpnea. Two recent findings have suggested a possible mechanism for neonatal chemoreception. In our studies of neurons from the LC, we find that these neurons are highly intrinsically chemosensitive during early development (P1–P10) and this response decreases substantially thereafter (Hartzler et al. 2007). Interestingly, a similar response has been reported for adrenal chromaffin cells (Muñoz-Cabello et al. 2005). In rats aged P1–P10, adrenal chromaffin cells respond to hypercapnia by releasing catecholamines, but lose this response to CO_2 after P10. Given that LC neurons are the major catecholaminergic brain region, we suggest that neonatal chemoreception involves hypercapnia-induced catecholamine release both systemically and within the brain. Such a response to hypercapnia would result in increased ventilation in a basic "fight or flight" response. This would represent a very simplistic but powerful and effective way to stimulate breathing in response to elevated CO_2. We further propose that this system wanes during the first week of life to be replaced by a more nuanced respiratory control system in adults. Another attractive feature of this model is that it predicts a critical window where the respiratory response to hypercapnia would be very weak during the transition from neonatal to adult chemoreception. Such a vulnerable period is consistent

with the epidemiological findings with sudden infant death syndrome, where the peak incidences occur 2–5 months after birth, not immediately after birth as one might predict for a linear developmental model of central chemoreception (Leiter and Bohm 2007).

Finally, it is not yet clear what factor(s) is/are responsible for the development of central chemoreception. This issue is complicated by the distributed nature of the central chemosensory neurons (see above). We have good evidence that, at least based on in vitro measurements, LC neurons decrease their intrinsic chemosensitivity with age during early development (Hartzler et al. 2007), while intrinsic chemosensitivity in NTS neurons is nearly fully developed at birth (Conrad et al. 2004, 2005; Putnam et al. 2005). The chemosensitive response of RTN neurons also does not appear to change throughout early development (Ritucci et al. 2005b) while medullary raphé neurons seem to increase their chemosensitivity with development. While these findings could be put together in a complex pattern to account for the triphasic pattern (or the linear pattern) of development of chemoreception, it is most likely that development of this important physiological regulatory system involves a complex pattern of development that includes the entire network and not just the development of the cellular response to CO_2 in neurons from a given chemoreceptive region of the brainstem.

8 Central Vs Peripheral Chemoreception

Classically, it has been assumed that the peripheral chemoreceptors, especially the glomus cells from the carotid bodies, serve a primary role increasing ventilation in response to hypoxia, and that the ventilatory response to CO_2 is largely mediated by central chemoreceptors (Dempsey 2005). This assumption was based on studies in which the central nervous system alone saw an acid challenge (Fencl et al. 1966; Heeringa et al. 1979) or ventral medullary neurons were ablated (Nattie et al. 1988). The findings in studies such as these suggested that the carotid bodies did not contribute, or at most contributed about 20–30% to the hypercapnic ventilatory response. However, it has been known for years that the glomus cells of the carotid body respond to hypercapnia as well as to hypoxia (Fidone and Gonzalez 1986), and thus it would stand to reason that they should sense and respond to CO_2/H^+ changes in the blood. A recent elegant study has re-investigated the role of the carotid bodies in the hypercapnic ventilatory response (Smith et al. 2006). In this study, one carotid body was denervated and the other had an isolated blood flow whose composition could be independently controlled. In such a preparation, the animal could be exposed to hypercapnia, both centrally (by increasing end tidal CO_2) and at the carotid body (by equilibrating the perfusion solution with hypercapnia), or to hypercapnia alone either centrally or at the carotid body. These studies clearly showed that only about 60% of the steady state increase in ventilation due to hypercapnia was due to central chemoreception and that the carotid bodies accounted for about 40%, a substantial fraction. More noteworthy, however, was the fact that the

ventilatory response to hypercapnia was delayed by about 11 s with central-only exposure as compared to exposure to both central and carotid bodies (Smith et al. 2006). The first finding indicates that the carotid bodies cannot be ignored when considering the effects of inspired CO_2 on ventilation, and that they can contribute a substantial proportion of an animal's ventilatory response to hypercapnia. The second finding suggests that the carotid bodies most probably play the major and determining role in rapid, breath-to-breath changes in blood CO_2/H^+ (Dempsey 2005; Nattie 2006). Such rapid and transient changes are seen in pathological conditions such as sleep apnea, where short periods of apnea (lasting several seconds) result in systemic hypoxia and hypercapnia. Under such conditions, it is the carotid body response that should be most responsible for generating the respiratory drive and it is within the carotid body that pathological changes are most likely to be observed. As proposed by Nattie (2006), this suggests that central chemoreceptors respond to changes in brain interstitial fluid CO_2/H^+ and would thus be sensitive to longer-term changes in systemic CO_2/H^+, as well as responding to changes in cerebral blood flow and/or metabolism. This would make the distributed network of central chemoreceptors a de facto system for indirectly monitoring brain oxygen levels, a concept that adds extra weight to our need to understand the precise signaling pathways in these chemosensitive neurons.

9 Cellular Chemosensitive Signaling

As discussed above, it is clear that there are numerous regions within the brainstem that, when focally acidified, result in increased ventilation. It has further been shown that in many of these areas, including the RTN (Mulkey et al. 2004; Ritucci et al. 2005b), the NTS (Dean et al. 1989, 1990a; Miles 1983), the LC (Filosa et al. 2002; Oyamada et al. 1998; Ritucci et al. 2005a), and the medullary raphé (Richerson et al. 2001; Wang et al. 2001), there are neurons whose firing rate responds reversibly to hypercapnia associated with decreased solution pH (elevated CO_2/H^+). [When an animal breathes air with elevated CO_2 or retains metabolically produced CO_2 (i.e., due to compromised lung function such as with chronic obstructive pulmonary disease), blood P_{CO2} rises with a fall of pH, and this condition is referred to as hypercapnic acidosis. It is mimicked experimentally by equilibrating artificial cerebral spinal fluid (aCSF) with high levels of CO_2, resulting in decreased pH (elevated H^+).] In this section we want to address the issue of how a neuron can respond to a change in CO_2/H^+, that is, what is/are the cellular signal(s) and target(s) that result in a change in firing rate of certain neurons in response to hypercapnia? The proposed models and the vast majority of the work has focused on neurons whose firing rate increases in response to increased CO_2/H^+, although in many regions there are a small percentage of putative chemosensitive neurons that respond with a *decrease* in firing rate in response to hypercapnic acidotic solution (Putnam et al. 2004). There are currently no good models for the cellular signaling in CO_2/H^+-inhibited neurons, but study of this process should provide interesting and novel

insights into neuronal responses to altered CO_2/H^+. We will focus our discussion on neurons whose firing rate is activated by increased CO_2/H^+.

Preparations for studying chemosensitive neurons. A number of preparations have been employed for the study of central chemosensitive neurons. In addition to in vivo studies of chemosensitive neurons (Mulkey et al. 2004), our understanding of the responses of these neurons to hypercapnic acidosis has been greatly facilitated by using reduced preparations. Four such preparations have been employed: the working heart-brainstem preparation, brain slices, organotypic cultures, and cell cultures. Each has distinct advantages and disadvantages that we will discuss here.

A recent example of in vivo preparations being used to study chemosensitive neurons from the rat RTN is the work of Mulkey et al. (2004). These rats were anesthetized with halothane and paralyzed, being artificially ventilated. The occipital plate was removed to allow a recording electrode to penetrate the medulla through the cerebellum. Recordings were made on groups of cells with extracellular electrodes. This technique allows for the study of neurons in near-physiological conditions with intact network connections and is thus presumably the closest measure to physiological conditions of any preparation. In fact, the argument has recently been made that in vivo measurements are needed before any neuron can be claimed to be involved in central chemosensitivity (Guyenet et al. 2008). However, such measurements are not without drawbacks. First, the use of anesthesia is known to alter the ventilatory response to inspired CO_2 (Akilesh et al. 1997) and it is clear that ventilatory control is state-dependent, differing during wakefulness, sleep and under anesthesia (Nattie 2000). Further, the use of halothane is particularly problematic since it is known to activate TASK channels, many of which are highly sensitive to changes in pH (Bayliss et al. 2001), and thus have been proposed to be a potential target of chemosensitive signaling (Mulkey et al. 2007). Other concerns about the use of anesthetized whole-animal preparations have been discussed (Nattie and Li 2006).

A reduced preparation that maintains many of the advantages of in vivo studies is the working heart-brainstem (Paton 1996) preparation. In this preparation, a mouse or rat is bisected at the level of the diaphragm, decerebrated, skinned, and the front limbs removed. A portion of the skull is removed to reveal the brainstem (the cerebellum is removed for dorsal exposures). The descending aorta is cannulated to enable the preparation to be perfused with warmed aCSF using its intact circulatory system. In addition to being more naturally perfused, this preparation benefits from having substantial amounts of the network intact, including peripheral chemoreception and cardiorespiratory reflexes, although input from higher brain centers is certainly lost. Another major advantage of this preparation is that no anesthesia is required. While access is limited for neurons within deeper centers, neurons near the surface have been studied with electrophysiological techniques (Paton 1996) and fluorescence imaging has been employed to study calcium transients within individual neurons (Bradley et al. 2008). As with in vivo preparations, the working heart-brainstem preparation requires considerable surgical skill and manipulation but has been used with both rats and mice.

The most widely used preparation to study neuronal chemosensitivity and chemosensitive signaling is the brainstem slice (Putnam et al. 2004). These studies involve the use of thin (300–400 μm thick) transverse or horizontal slices of various brainstem regions. Slices allow excellent access to individual neurons from either neonates (Filosa et al. 2002; Ritucci et al. 2005a, b) or adults (Dean et al. 1989, 1990a; Nichols et al. 2007) and excellent control over the extracellular solution. Further, signaling within these neurons can often be studied using fluorescence imaging microscopy (Filosa et al. 2002; Ritucci et al. 1996, 1997, 2005b), adding to our ability to study cellular chemosensitive signaling mechanisms. Individual neurons within the slice can also be voltage-clamped, allowing for the study of potential ion channel targets of chemosensitive signaling (Mulkey et al. 2007). These preparations can often be studied for hours and are not usually exposed to anesthetics. Indeed, much of what we have learned about cellular chemosensitive signaling over the past 15 years has derived from the use of brainstem slices (Putnam et al. 2004).

The slice preparation, however, has significant limitations. While the local network is maintained somewhat intact, there is a substantial loss of the overall network, including inputs from higher centers and from brainstem regions farther removed than a few hundred microns. There are also superfusion limitations, and the administration of drugs can take some time to diffuse into the tissue to the neuron being studied, potentially altering the kinetics of the response being studied (Ritucci et al. 2005a). The diffusion issue has recently introduced another concern about the slice preparation. Traditionally, because of the thickness of the slice, superfusion solutions are equilibrated with 95% O_2 to avoid hypoxia in the core of the slice. However, it has been shown recently that this results in a considerable hyperoxia compared to normal brain tissue in an animal breathing room air (P_{O_2} of 10–35 Torr) (Dean et al. 2003; Mulkey et al. 2001). This hyperoxia occurs even in the core of the slice, where P_{O_2} can be as high as 300–500 Torr, depending on superfusion conditions (Mulkey et al. 2001; Potter et al. 2004). Further, hyperoxia has been shown to activate some brainstem neurons, increasing their firing rate, and this activation occurs preferentially in CO_2-sensitive neurons within the NTS (Mulkey et al. 2003). This activation may be related to the accumulation of reactive oxygen and/or nitrogen species in response to hyperoxia, which have been shown to activate chemosensitive NTS neurons (Lipton et al. 2001). In fact, recent findings indicate that slices exposed to 95% O_2 will rapidly accumulate reactive oxygen species and show greater cell death than slices maintained at 60% O_2 (D'Agostino et al. 2007). Thus, it is likely that the typical slice preparation is a highly activated preparation due to the use of hyperoxic conditions, and the actual response of central chemosensitive neurons to hypercapnia, as studied with brain slices, will require a re-investigation of the proper level of control O_2 to be used.

A variant of the slice experiment has been used by some laboratories (Wiemann and Bingmann 2001). These studies employ organotypic cultures, where a slice (300–400 μm) is placed into organ culture for several weeks. The slices tend to flatten to only about 100–200 μm thick and individual neurons at the edge of the organotypic culture are easy to visualize, facilitating impalement and imaging of these neurons. In addition, these neurons maintain associations with other neurons

and with glia, and remain sensitive to CO_2 (Wiemann and Bingmann 2001). The major disadvantage to the use of these slices is the concern about changes in neuronal properties and local network properties over time in culture, and the same concerns about the lack of a fully intact network that were mentioned above for acute slices.

Finally, central chemosensitive neurons have been studied in cell culture. Hypercapnia-stimulated neurons have been cultured from the ventrolateral medulla (Rigatto et al. 1994), the medullary raphé (Wang et al. 1998), and recently the LC (Johnson et al. 2008). These neurons generally need to be in culture for over a week before they develop a chemosensitive response and are often studied after 2–4 weeks of being in culture. These neurons give a robust and repeatable response to hypercapnia, which is often larger than their response in slices (compare Wang et al. 1998 with Mulkey et al. 2007 and Johnson et al. 2008 with Filosa et al. 2002). The culture preparation is ideal for studying chemosensitive signaling because the environment can be readily controlled and rapidly altered, and there is easy access to the neurons for electrophysiological or imaging studies. Further, these preparations should prove useful for genetic manipulation such as siRNA knock-down experiments which will allow for direct testing of chemosensitive signaling models. As always, there are concerns about the use of cultured cells. The main concern is whether the physiology of the cell has been altered by its prolonged period in culture. For instance, cultured neurons have been shown to alter their expression of pH-regulating transporters compared to the same neurons that are freshly dissociated (Raley-Susman et al. 1993). As mentioned above, the magnitude of the firing-rate response to hypercapnia seems to be higher for neurons from the same brainstem region when in culture compared to in-the-brainstem slice. Thus, while cell culture will undoubtedly prove effective in studying the cellular basis of chemosensitive signaling, the results from such experiments will have to be confirmed in brain slices, and ultimately in vivo, before we can be assured that the described mechanism is physiological or not.

Chemosensitive signaling. The working model for chemosensitive signaling has been that hypercapnia results in an influx of CO_2 into a chemosensitive neuron, resulting in a fall of intracellular pH (pH_i) (Lassen 1990; Putnam et al. 2004). This fall in pH_i is believed to inhibit a K^+ channel, resulting in neuronal depolarization and increased firing rate. A hypercapnia-induced maintained fall of pH_i has been observed in many chemosensitive neurons (Filosa et al. 2002; Putnam et al. 2004; Ritucci et al. 1997; Wiemann and Bingmann 2001). This hypercapnia-induced fall of pH_i is correlated, both kinetically and in magnitude, with the increased firing rate in LC neurons (Filosa et al. 2002), suggesting that the change of pH_i is an important intracellular signal in chemosensitive cells. However, the correlation of the change of pH_i and increased firing rate is not perfect and is very poor upon removal of hypercapnia (Filosa et al. 2002). Further, a poor correlation between hypercapnia-induced acidification and the increased firing rate is seen in RTN neurons (Ritucci et al. 2005b). These findings suggest that the firing rate response of a chemosensitive neuron to hypercapnia is determined by more than the fall of pH_i. This was directly demonstrated in experiments in which LC neuron pH_i was clamped at a constant value during exposure to hypercapnic acidosis. Under these conditions,

hypercapnia was still able to induce an increase in firing rate of LC neurons, clearly demonstrating that a fall of pH_i is not a necessary signal for chemosensitive signaling (Hartzler et al. 2008b). Interestingly, Hartzler et al. (2008b) were able to show that when LC neurons were exposed to hypercapnia with both pH_i and external pH (pH_o) constant, firing rate did not increase. These data suggest that LC neurons are responding to changes of pH, either pH_i or pH_o.

There are several other factors that may be involved in the chemosensitive response of neurons (Putnam et al. 2004). The firing rate response of LC neurons to hypercapnic acidosis has been shown to be reduced by half by the L-type Ca channel inhibitor nifedipine (Filosa and Putnam 2003), indicating a role for Ca channels and/or intracellular Ca in chemosensitive signaling. As mentioned above, the production of reactive oxygen species can activate some chemosensitive neurons, indicating that the redox state may also serve to modulate chemosensitivity. Other factors, which undoubtedly contribute to the neuronal chemosensitive response, include neurotransmitters, glial cells and carbonic anhydrase activity (see Putnam et al. 2004).

Just as it appears that more than simply changes of pH_i are involved in chemosensitive signaling, there is unlikely to be a single chemosensitive channel that is inhibited by hypercapnia. Several pH-sensitive ion channels could be candidate targets for chemosensitive signaling (Putnam et al. 2004). There is, in fact, evidence for the involvement of several ion channels in chemosensitive signaling, especially in LC neurons. In addition to the activation of L-type Ca channels by hypercapnic acidosis (Filosa and Putnam 2003), the K channel inhibitor 4 aminopyridine (4AP) partially inhibits the increased firing rate induced by hypercapnic acidosis in LC neurons (Martino and Putnam 2007). A similar effect is seen with another K channel inhibitor, tetraethyl ammonium (TEA), which reduces hypercapnia-induced depolarization of LC neurons (Filosa and Putnam 2003). Finally, decreased pH_o also appears to be involved in the chemosensitive response of LC neurons (Filosa and Putnam 2003; Hartzler et al. 2007), suggesting the involvement of TASK channels in chemosensitive signaling (Bayliss et al. 2001). Thus, the response of LC neurons to hypercapnia appears to involve numerous channels, including L-type Ca channels, TASK channels, and 4AP- and TEA-sensitive K channels.

The plethora of signals and ion channel targets involved in chemosensitive signaling lead us to propose a new model for chemosensitive signaling in LC neurons, the multiple factors model (Putnam et al. 2004). In this model, the LC neuronal response to hypercapnic acidosis is not envisioned to be the result solely of changes of pH_i inhibiting a single K channel. Rather, multiple signals (changes of pH_i and pH_o, opening of Ca channels and perhaps increased intracellular Ca) affect multiple ion channels (Ca channels, TASK channels and various K channels), and the final firing rate of an LC neuron in response to hypercapnia is the result of all of these effects.

Finally, since central chemosensitivity is a distributed property, we must ask whether multiple ion channels are involved in the response to hypercapnia of neurons from all chemosensitive areas. While we know less about the signaling processes in neurons from other chemosensitive regions, the answer appears to be

that multiple ion channels are not involved in the response of chemosensitive neurons from all regions. For instance, a single K channel inhibitor, 4AP, is sufficient to fully block the increased firing rate response to hypercapnia in NTS neurons (Dean et al. 1990a; Martino and Putnam 2007). In mice that have had TASK-1, TASK-3 or both channels knocked out, hypercapnia no longer activates medullary raphé neurons (Mulkey et al. 2007). In these same knockout mice, however, the response of RTN neurons to hypercapnia is unaffected (Mulkey et al. 2007). Thus, in the NTS, medullary raphé and perhaps the RTN, a single ion channel seems to be responsible for chemosensitive signaling, although it appears to be a different ion channel in each area. This latter observation suggests that chemosensitivity arose independently in the neurons from each region and that the distributed network of central chemosensitivity had a polyphyletic origin.

10 Summary

Understanding of the mechanisms of central chemosensitivity has been greatly expanded (Remmers 2005), yet there are several pertinent questions that have not been answered (Gaultier and Gallego 2005). Why are there multiple chemosensitive sites in the brainstem? How do these sites interact with one another? Do any of the sites have more or less control under varying circumstances? Much of our understanding of central chemosensitivity comes from work done in typical model organisms, yet one of the tools to address these questions is the use of the Krogh principle (Krogh 1929) where for any physiological problem there is/are some animal(s) best suited to address that problem. In this chapter we have described several situations (e.g. hibernation, torpor, fossorial habitats, diving) where a number of mammalian species undergo extreme changes in their ventilation as part of their normal life history. To date, the potential for gathering information regarding central chemosensitivity in these organisms has not been exploited.

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