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HIGHLIGHTED TOPIC | Central CO_2 Chemoreception in Cardiorespiratory Control

Central chemoreception in wakefulness and sleep: evidence for a distributed network and a role for orexin

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Nattie E, Li A. Central chemoreception in wakefulness and sleep: evidence for a distributed network and a role for orexin. *J Appl Physiol* 108: 1417–1424, 2010. First published February 4, 2010; doi:10.1152/japplphysiol.01261.2009.—This minireview examines data showing the locations of central chemoreceptor sites as identified by the presence of ventilatory responses to focal, mild acidification produced in unanesthetized animals in vivo, how the site-specific responses vary by arousal state, and what the emerging role of orexin might be in this state-dependent central chemoreceptor system. We comment on the organization of this distributed central chemoreceptor system and suggest that interactions among sites are synergistic and not additive, which is an important aspect of its normal function.

control of breathing; carbon dioxide; pH; systems physiology

CENTRAL CHEMORECEPTOR SITES IDENTIFIED BY VENTILATORY RESPONSES TO MILD FOCAL ACIDIFICATION IN VIVO

CENTRAL CHEMORECEPTION (CCR) refers to the detection of CO_2/pH at sites within the central nervous system and the resultant effects on ventilation. We recently summarized data showing the inhibitory effects on CCR of lesions or focal inhibition at three putative central chemoreceptor sites, the retrotrapezoid nucleus (RTN), the medullary raphe (MR), and the locus ceruleus (LC) as well as of lesions of neurons in the ventral medulla that express neurokinin-1 receptors (NK1R) (54). Such studies do not necessarily identify sites or neurons that detect CO_2/pH but do identify sites or neurons that participate in CCR. Here we review data showing *1*) locations of central chemoreceptor sites identified by the presence of ventilatory responses to focal, mild acidification produced in vivo; *2*) how these responses vary by arousal state; and *3*) what the role of orexin is in this CCR system. We further comment on the issue of the organization of central chemoreception as a unique single site as opposed to a widespread distribution of sites that vary in function with arousal state. This paper will not consider the nature of the primary stimulus, i.e., whether it is CO_2 or pH, or if pH, whether it is intracellular or extracellular pH, but will refer to this stimulus as CO_2/pH. We use the term "focal acidification" in this review without precluding the possibility that CO_2 per se might act as a stimulus.

The phenomenon of CCR was discovered in the 1950s when an increase in breathing was noted following perfusion of acidic solutions within the brain (35, 40, 76). Subsequent work localized CO_2/pH detection to the ventral medullary surface at rostral (Mitchell's) (46) and caudal (Loeschcke's) (44) "chemosensitive" areas with an area in between which while not chemosensitive did have profound effects on breathing, the intermediate (Schlaefke's) area (75). The localization of central chemoreception to named areas on the ventral medullary surface has had a persistent influence on those interested in the chemical control of breathing. However, even at the time of their discovery there were those who proposed a wider distribution of CCR sites. Their main arguments were twofold. First, the intensity of the acidic stimulus applied to the ventral surface in these early experiments was severe (49). Second, sites responsive to pH changes could be identified at some depth from the ventral surface (49).

In 1965, John Pappenheimer and colleagues provided new insight into the problem by performing a series of experiments in conscious goats (20, 65). Prior experiments had been performed largely under anesthesia, which *1*) enhances the brain tissue pH response to a given systemic CO_2/pH challenge, and *2*) considerably depresses the ventilatory response to CO_2/pH. In conscious goats with chronic acid-base imbalance, they performed ventriculo-cisternal perfusion using artificial cerebrospinal fluid (aCSF) with varying bicarbonate concentrations along with changes in inspired CO_2 and correlated blood and CSF acid-base status with ventilation. They deduced that steady-state ventilation was uniquely determined by brain interstitial pH at a location two-thirds of the distance between average capillary blood and large-cavity CSF, a virtual location not an anatomic one. The studies showed a very high sensitivity of the CCR response obtained in these conscious animal experiments.

In 1993, we observed that 1-nl injections of acetazolamide (5–10 μM) into the brain stem of anesthetized cats and rats in vivo decreased tissue pH in a focal, localized manner (11). By

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applying a tissue pH electrode we showed that the stimulus intensity was like that associated with a 36-mmHg increase in arterial P_{CO_2} and the region of decreased pH was limited to within 350 μm from the center of the injection. We used this finding to probe for CCR sites. At some injection locations these injections increased breathing while at others they did not. The CCR sites uncovered using this experimental approach by us (11) and others included *1*) within 800 μm of the ventrolateral medullary surface at locations dorsal to the traditional rostra1 and caudal chemosensitive areas and the intermediate area, *2*) the nucleus tractus solitarii, *3*) the locus ceruleus, *4*) the rostral aspect of the medullary raphe (raphe magnus) (5), *5*) the pre-Bötzinger complex (77), and *6*) the fastigial nucleus of the cerebellum (45). These experiments had two weaknesses. First, the stimulus intensity, although focal within the brain, was large, and second, there was the possibility that acetazolamide affected variables other than pH. Nevertheless these data suggested the presence of a widespread distribution of CCR sites that can affect breathing in the anesthetized animal in vivo.

In 1999 (42) we published the first of an ongoing series of experiments in unanesthetized animals in which we produced a focal acidosis by reverse microdialysis of aCSF (bicarbonate concentration of 26 mM) equilibrated with high CO_2. Under anesthesia, we found that dialysis with an aCSF equilibrated with 25% CO_2 reduced tissue pH measured by microelectrode in situ by an amount like that found with an increase in arterial P_{CO_2} of 35 mmHg and that the region of acidosis was limited to within 550 μm of the probe tip (42). Subsequently, in conscious rats we showed that a similar dialysis in the retrotrapezoid nucleus (RTN) decreased focal pH measured within 200 μm of the dialysis probe by much less, an amount equivalent to that induced by a 6.6-mmHg increase in arterial P_{CO_2} (41). Dialysis with aCSF equilibrated with 5% CO_2 did not change brain pH. While it is possible that there could be a steeper drop in pH between the probe surface and the pH electrode, it is important to note that the diameter of our modified Beetrode pH electrode is itself 100 um so it is challenging to get it much closer than 200 μm. Further, the Forster lab (28) has shown that when pH is measured in 100 μl of an aCSF solution in vitro with dialysis using a probe the same size as on our experiments the pH decreased by ~14% of that in the dialysate. Further they also compared the pH change produced by dialysis of aCSF equilibrated with different concentrations of CO_2 in vivo and in vitro. The pH change in vitro would reflect that adjacent to the dialysis probe as well as throughout the solution while that in vivo would reflect also the powerful "buffer" effect of local cerebral blood flow. The measured pH change in vivo was much smaller than that in vitro, on average ~20% of the in vitro change. The difference in the degree of the brain tissue pH response to the same dialysis conditions in anesthesia vs. in the conscious rat underscores the importance of cerebral blood flow and focal ion-exchange responses in the tissue response to any given degree of CO_2/pH change (1), i.e., under anesthesia cerebral blood flow is less and clearance of the focally dialyzed high CO_2 is less.

Our application of this technique in the conscious, unanesthetized rat allows examination of focal central chemoreceptor sites using a physiologically relevant stimulus applied directly within the brain at a moderate intensity, one that is equivalent to a 6.6-mmHg increase in arterial P_{CO_2}, and it allows studies in sleep and wakefulness. It is important to note that this stimulus is applied directly at the CCR site of interest, the peripheral chemoreceptors are not directly affected, and that the stimulus intensity is considerably milder than that associated with using 5% or 7% inspired CO_2 as a CCR stimulus. When breathing 7% CO_2 the arterial P_{CO_2} in the conscious rat increased by 15 mmHg and the degree of brain tissue acidosis was double that observed with focal acidification by dialysis (41).

To date, six CCR sites have been described using this microdialysis approach. *1*) Focal acidification of the RTN in the rat increased ventilation ~24% due to increases in tidal volume (42). *2*) In the rostral medullary raphe (MR), focal acidification by dialysis induced an ~20% increase of ventilation mediated by respiratory frequency in the rat (56). *3*) Focal acidification in the caudal MR (raphe obscurus) alone had little effect on ventilation in the rat but if performed simultaneously with focal acidification of the RTN, the response was much greater (51% increase) than focal RTN acidification alone (24% increase) (13). Thus the caudal MR can detect CO_2/pH but the ventilatory response requires an interaction with the RTN. Hodges et al. (28, 29) used this technique to focally acidify the MR in conscious goats. They found that focal stimulation of the more caudal aspect of the MR increased ventilation, findings somewhat different from ours, which might be explained by their use of a greater stimulus intensity, being like that associated with breathing 7% CO_2. *4*) Focal acidification of the caudal nucleus tractus solitarius (NTS) of the rat increased ventilation by 20–30% (55). *5*) Focal acidification of the region just dorsal to the caudal ventral medullary surface chemosensitive area in rat significantly increased ventilation by ~17% (da Silva, Li, and Nattie, unpublished observations). *6*) Focal acidification of the pre-Bötzinger region increased ventilation by 10% in conscious goats (36). Thus a mild focal acidosis at many, but not all, brain stem sites can produce a stimulation of ventilation.

We can estimate in a very approximate way the contribution of each of these central chemoreceptor sites to the overall response by assuming a linear additive interaction. If all sites, plus the carotid body, are exposed to a ~6.6-mmHg increase in arterial P_{CO_2} in the unanesthetized rat, ventilation increases by ~120% (7, 38, 71). If we sum the isolated responses to focal acidification of the RTN, caudal NTS, and caudal chemosensitive area in wakefulness we find a total of a ~67% increase, somewhat less than the estimated 120% increase. If we add the increased efficacy of RTN acidification when the caudal MR is simultaneously acidified we estimate a response total of a 94% increase, still short of 120%. The discrepancy between 94% and 120% can in part be accounted for by consideration of the fact that the increase in ventilation resulting from focal acidification lowers the arterial P_{CO_2} and this hypocapnia inhibits other chemoreceptor sites (41). Further it seems likely that there is greater synergism when many sites are stimulated simultaneously as demonstrated by focal acidification of both RTN and caudal MR (13). And there are, in our view, many sites including, in addition to those mentioned above, the locus ceruleus (LC) (6, 63), the fastigial nucleus of the cerebellum (45), the pre-Bötzinger complex (36, 77), and the orexin neurons (12). Thus the CCR system may not behave in a simple additive manner; there is likely considerable interaction among CCR sites and with the carotid body inputs as well.

Of course, this technique has its concerns. The placement of a probe into the brain stem unavoidably produces tissue damage. The dialysis could be affecting other unmeasured variables. It is a difficult technique to perform. It requires excellent small-animal surgical skills to quickly and reliably place the probe guide tubes into the proper locations, and the experiments are tedious in that the rat must be studied under calm, quiet conditions with long periods of quiet wakefulness and sleep absent of exploration and sniffing during the dialysis. Care must be used in the handling of CO_2 as it is highly soluble and diffuses easily into the atmosphere from solutions and tubing. To alleviate some of these concerns we always include probe placements that have no response to the test dialysis as well as control dialysis with aCSF equilibrated with 5% CO_2 at each site. Two further concerns have been raised (9, 27). *1*) What does topical acidification of a brain region in vivo mimic? In that brain tissue pH responses vary at different brain stem sites in response to a brief basilar artery infusion of CO_2 enriched saline (4, 34), could our focal acidification be affecting regions that would not normally be acidified in response to a systemic stimulation? This seems highly unlikely given the wide distribution of sites that do acidify following the vertebral artery injection of CO_2 laden saline [see Fig. 6, Arita et al. (4)]. Further, the data of Arita et al. (4, 34) showing site-dependent pH responses to brief CO_2/pH stimulation were obtained in anesthesia; whether such tissue pH heterogeneity exists in the conscious animal seems less likely given the substantial difference in focal tissue pH that we have measured following dialysis with CO_2-rich aCSF in anesthesia vs. the awake state (vida supra). We conclude that focal acidification mimics changes in brain CO_2/pH that occur in the unanesthetized rat when arterial P_{CO_2} changes or when brain pH changes due to alterations in cerebral blood flow or metabolism. *2*) Could the focal region of acidification involve terminals from distant neurons, e.g., RTN or raphe serotonergic neurons or other? The focal dialysis approach, as currently applied, does not allow the distinction of whether a cell body or cell process is being stimulated or for that matter whether it is a neuron or glial cell. However, the effects on breathing components, i.e., tidal volume vs. frequency, and the arousal state in which they occur often differ by site. For example, if we examine the two CCR sites in closest proximity, the RTN and the rostral MR, we find that focal acidification of the RTN affects tidal volume predominantly and does so in wakefulness (41) while focal acidification of the rostral MR increases breathing frequency predominantly and does so in sleep (56). And in the LC studied as a slice preparation a direct application of an acidic stimulus affected the neuronal soma, not its dendrite (69a). In other sites, however, we cannot by the application of focal acidification by microdialysis be certain what structures are being stimulated. We can be certain that a specific region is being stimulated and that information alongside data from other studies, e.g., demonstrating that RTN or medullary raphe 5-HT neurons are chemoresponsive in reduced preparations, allows the reasonable deduction that there exists a wide distribution of separate CCR sites.

Remarkably, during focal dialysis the rats cycle through sleep and wakefulness allowing detection of changes in ventilation in both states while a single brain region is acidified [see Fig. 1 in Ref. 55 and Fig. 1 in Ref. 56 to examine tracings obtained during focal acidification in the MR and RTN that demonstrate, during the period of focal acidification, rapid changes in ventilation when arousal state changes]. Using this technique we have discovered that the small but significant increases in breathing at the sites studied to date occur in an arousal state-dependent manner: the responses within the RTN, caudal NTS, and caudal medullary surface region are in wakefulness; the responses in the MR and caudal NTS are in sleep. {The response to focal acidification of the MR in the goat occurred in wakefulness not sleep [Hodges et al. (28)]. This could reflect a species difference or a difference in focal stimulus intensity as their focal pH change was more like that associated with breathing 7% CO_2, a value more acidic that that observed in the rat.}

We conclude that these focal acidification results provide strong evidence in support of the hypothesis that CCR, at least as it applies to breathing, has sensing sites that are widely distributed in the brain stem (19, 50, 51, 54, 62). Further, we conclude that the function of some sites varies with arousal state and, when considered in the light of studies performed in vitro showing CO_2/pH responses in many different cell types (6, 18, 47, 68), that different cell types participate in vivo.

OREXIN, AROUSAL AND CENTRAL CHEMORECEPTION

We consider here the role of orexin in CCR as a beginning in understanding how chemoreception might be linked mechanistically to arousal state. There are two orexin subtypes, orexin-A and orexin-B, derived from the same precursor (prepro-orexin), which bind to two G protein-coupled receptors: orexin receptor-1 (OX_1R), selective for orexin-A, and orexin receptor-2 (OX_2R), responsive to both orexin-A and -B. The neurons that contain orexin are located solely in the dorsal and lateral hypothalamus and perifornical area, have widespread projections, and are associated with multiple functions, such as control of energy homeostasis, feeding behavior, reward processes, sleep-wake states, stress response, nociception, and cardiovascular and respiratory control (66, 72–74, 88).

Orexins play a key role in the control of vigilance, and diminished orexin function can result in a specific clinical syndrome, narcolepsy, in animals and humans. Orexin neurons have state-dependent activity (39), have excitatory inputs to nuclei that regulate arousal (64, 66), and are linked to the control of breathing (17, 23, 37, 48, 86). With respect to CCR, orexin neurons are activated by CO_2/pH in vitro (84) and in vivo as measured by c-fos activation (79), and prepro-orexin knockout mice have a 50% decrease in the ventilatory CO_2 response during wakefulness, an effect that is reversible by administration of orexin-A and orexin-B (12, 37, 48). Administration via the cerebral ventricles of an OX_1R-selective antagonist (SB-334867) decreases the respiratory chemoreflex by 24% in mice (12).

We have begun a series of experiments to ask which CCR sites are affected by orexin and whether the effects at these sites vary with arousal state and the diurnal cycle. At the RTN region, where there is evidence for OX_1Rs, we dialyzed an OX_1R antagonist, SB-334867, in unanesthetized rats during a study time that included the end of the light, inactive period and the beginning of the dark, active period. We observed a 30% reduction of the ventilatory response to breathing 7% CO_2 that occurred during wakefulness, while during non-rapid eye movement (NREM) sleep the inhibitory effect was much

smaller, a 9% reduction (14). We did not attempt to separate these data by diurnal cycle.

As orexin levels vary across 24 h, with higher levels in the dark, active period compared with the light, inactive period (85), we examined the effects of the diurnal cycle on orexin receptor antagonism and CCR. We inhibited neurons in the rostral MR, which receives projections from orexin-containing neurons, by focal microdialysis of SB-334867 during the dark, active and, separately, during the light, inactive periods of imposed diurnal cycles (14a). During wakefulness in the dark period, but not in the light period, OX_1R antagonism caused a 16% reduction of the ventilatory response to 7% CO_2 compared with vehicle. There was no significant effect in sleep. The basal ventilation, body temperature, and oxygen consumption ($\dot{V}O_2$) were not affected. No effect was observed in a separate group of animals that had the microdialysis probe misplaced (periraphe).

To examine the effect of widespread pharmacological blockade of both orexin receptors, we administered by oral gavage Almorexant, an antagonist of both OX_1R and OX_2R generously supplied by Actelion Pharmaceutical. This drug has been shown to promote sleep in animals and humans (10). Here we asked if systemic administration of this dual orexin receptor antagonist would affect the CO_2 response. Each rat was tested in the inactive and active phase 2 h after administration by oral gavage of Almorexant (300–400 mg/kg) or control vehicle. We verified that Almorexant, used by us at a slightly higher dose compared with the original paper (10), decreased the amount of time spent in wakefulness and increased the time spent in NREM sleep during the dark, active period with little significant effect on wake-sleep times during the light, inactive period. Compared with the control in the same diurnal phase, Almorexant decreased rat body temperature independent of diurnal cycle and, during the active phase of the diurnal cycle, decreased oxygen consumption, presumably by decreasing activity, and decreased the CO_2 response normalized to metabolic rate ($\dot{V}E/\dot{V}O_2$, where $\dot{V}E$ is minute ventilation; −16% in wakefulness, −15% in NREM sleep). The inhibitory effects on the CO_2 response were present only in the active phase of the diurnal cycle. Given the 50% decrease in the CO_2 response observed in the orexin null mice, we were puzzled by the small effect on the CO_2 response produced by the systemic administration of this dual-receptor antagonist. One explanation may be that the dose required to inhibit CCR sites may be higher than that used. A second explanation may be that chemoreception involves preferentially OX_1R. Recent work indicates that Almorexant binds with slightly greater efficacy at OX_2R than OX_1R (16) and that OX_2R may be involved to a greater extent in the wake-promoting effects of orexin (16).

The state-dependent aspect of the role of orexins in CCR complements the growing evidence that orexin neurons are essential to promote and consolidate wakefulness (23). Our data provide some support for the notion that the orexin system may play a key role in the "wakefulness drive" of breathing (14, 23). Our results with focal RTN OX_1R antagonism did show a decrease in ventilation during air breathing (14), suggesting the possibility that orexin provides a portion of the "wakefulness drive." However, our findings with focal OX_1R antagonism in the MR did not support this (14a). Deng et al. (12) found that the intracerebroventricular (icv) administration of SB-334867 did not influence spontaneous ventilation in mice, whether in wakefulness, or in sleep. And our findings with antagonism of both OX_1R and OX_2R using systemic Almorexant did not show hypoventilation in air breathing in wakefulness. The role of orexin in the "wakefulness" drive is uncertain.

Of added interest with respect to the functions of orexin in the control of breathing are the observations that under anesthesia, orexin neuronal activity is decreased by anesthetic agents also associated with depression of respiration but not by agents that do not depress breathing (87). Further, the recovery time after anesthesia is prolonged with orexin inhibition (33). With greater knowledge of OXR subtypes involved in wakefulness (OX_2R?) vs, chemoreception (OX_1R?), perhaps a receptor-specific agonist could be used to minimize respiratory depression during and just after anesthesia.

CENTRAL CHEMORECEPTION IN SLEEP AND WAKEFULNESS

Figure 1 summarizes our current thinking of central chemoreception during wakefulness, Fig. 2 for NREM sleep. The solid lines between various structures show known functional connections; the dashed lines show anticipated but as yet untested functional connections. The unshaded areas are CO_2 responsive by in vivo focal acidification testing in unaesthetized animals. In wakefulness we propose a system in which specific orexin inputs to the RTN and rostral MR enhance the overall sensitivity of the system, which includes responses that originate at the RTN, caudal NTS, the carotid bodies (CB), and likely the LC. The caudal MR can affect the CO_2 response indirectly via an amplification of the response at the RTN (14, 43). The newly described responses to focal acidification of the caudal ventrolateral medulla (VLM) may affect the system response via the RTN and/or the central pattern generator (CPG). While many inputs exist to the RTN from other sites, so too are there many inputs directly to the CPG. It is unclear at present how the various CCR sites proportion their efferent activity between the RTN and the CPG. Further it is possible that orexin inputs may affect CCR responses at sites other than the RTN and the rostral MR.

Figure 2 shows our current view of central chemoreception in NREM sleep. Orexinergic excitation of CCR is absent. The main sources of currently known chemoreception at the stimulus levels obtained by our in vivo dialysis approach are the caudal NTS, rostral MR, and CB. The role of the LC in sleep is unknown. We do not know if there is any synergy between CCR sites during sleep.

IS THERE A SINGLE DOMINANT CENTRAL CHEMORECEPTOR SITE?

Recent debate has centered on two sites and their associated neurons as being of primary importance in CCR, the RTN and the MR. The Guyenet laboratory has published a series of studies performed under anesthesia showing that the RTN neurons that are involved in CCR have a specific phenotype, they express the transcription factor Phox2b as well as VGlut2, and they project to multiple sites involved in the control of breathing (25–27, 47, 70, 78, 81). These RTN neurons receive inputs from the carotid body (80) and from the hypothalamus (22). Thus they may act as chemodetectors and as integrators of afferent information (27). Strategies to discern the importance of these RTN neurons by lesion, inhibition, or focal

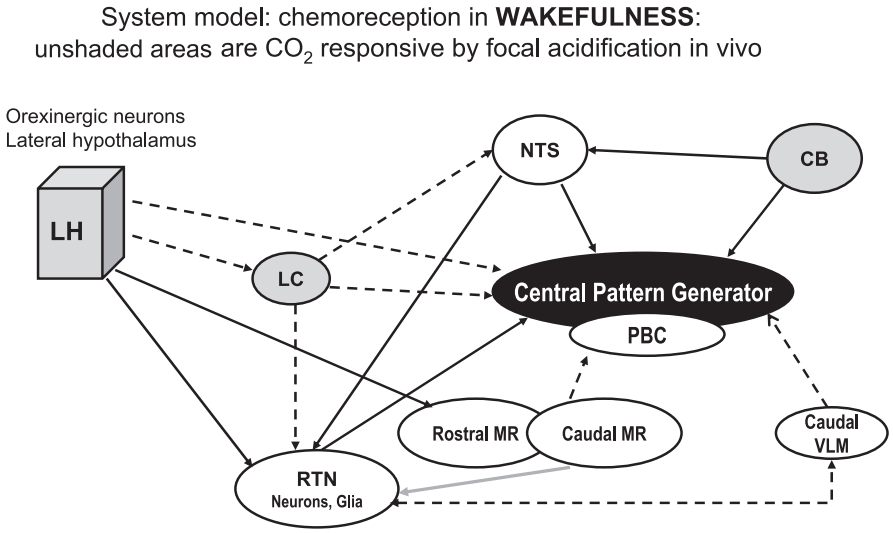

Fig. 1. Schematic model for central chemoreception in wakefulness that represents our current working hypothesis. The unshaded areas represent sites at which focal acidification by dialysis with artificial cerebrospinal fluid (aCSF) equilibrated with high CO_2 produced an increase in ventilation in wakefulness. The areas in gray represent sites at which we anticipate a response to focal acidification in wakefulness. Solid lines show established functional connections related to chemoreception, e.g., dialysis of an orexin receptor 1 (OX_1R) antagonist at the retrotrapezoid nucleus (RTN) decreased the CO_2 response in wakefulness. Dashed lines show likely connections that remain to be established. That linking the caudal medullary raphe (MR) to the pre-Bötzinger complex (PBC) reflects observations obtained in a slice preparation (66a). The gray line between the caudal MR and the RTN reflects a CO_2-linked connection, i.e., focal acidification of the caudal MR enhances the response to focal acidification of the RTN. LH, a designation that includes orexin neurons in lateral hypothalamus, dorsomedial hypothalamus, and perifornical area; LC, locus ceruleus; CB, carotid body; NTS, nucleus tractus solitarius; VLM, ventrolateral medulla. See text for references.

stimulation suffer from two major problems: *1*) the small number of RTN neurons exist as a thin sheet lying between the facial motor nucleus and the ventral medullary surface, a difficult area to target; and *2*) given the number of emerging roles that can be attributed to these neurons it is difficult by lesion, inhibition, or stimulation to interpret which function has been altered as the primary effect.

One argument for the primary role of the RTN in CCR emerges from analogy with the human central congenital hypoventilation syndrome (CCHS), which has, among many dysfunctions, a decreased response to CO_2 especially in sleep and a strong genetic association with mutations in the transcription factor, PHOX2B (83). The prominent role proposed for Phox2b-expressing neurons within the RTN in chemoreception (vida supra) along with the fact that mice with polyalanine Phox2b genetic expansion have absent RTN Phox2b neurons and have a phenotype of abnormal breathing at birth, a diminished response to CO_2, and rapid death (3, 15) have been parlayed into the argument that RTN Phox2b neurons are the primary central chemoreceptor site, a revisiting of the old VLM surface primacy hypothesis. The recent evidence that the RTN/parafacial respiratory group (pFRG) neurons are involved in rhythm generation in early embryonic life (21) and the fact that the polyalanine expansion genetic model for CCHS results in immediate postnatal death suggest that caution should be used in extending to adults an importance for RTN Phox2b neurons based on findings obtained in the embryo or neonate. There is little doubt that neurons and perhaps glia (24) within the RTN participate in CCR as inhibition by muscimol (52), lesions by kainic acid (2, 57, 60, 61) and by specific killing of

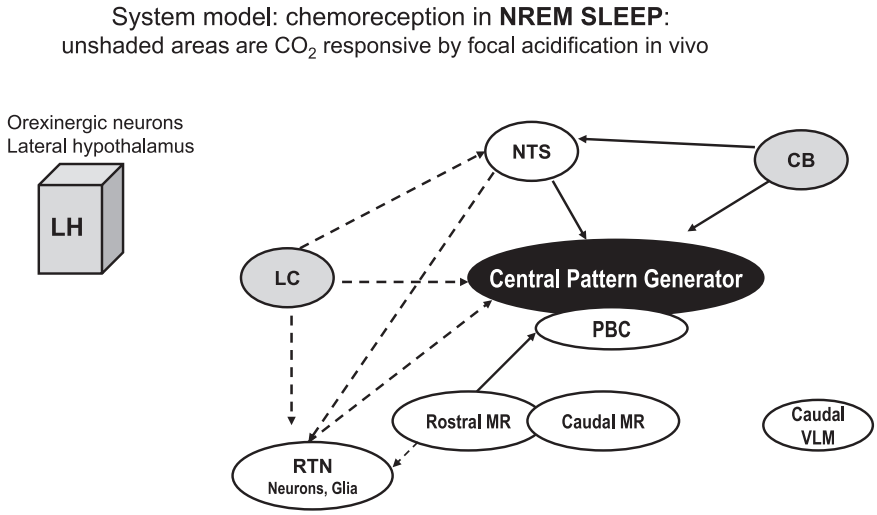

Fig. 2. Schematic model for central chemoreception in non-rapid eye movement (NREM) sleep that represents our current working hypothesis. The symbols and lines are as in Fig. 1.

NK1R-expressing cells (53, 58) all reduce the CO_2 response in conscious rats and focal acidification increases ventilation (41). At issue is the role of RTN neurons in: *1*) chemoreception (Do they detect CO_2/pH? There is no evidence to date that they are intrinsically responsive to CO_2/pH but they do respond in situ. Are they the sole physiologically relevant CO_2/pH detector?); *2*) rhythm generation in early life [Are they the first rhythm generator in embryonic life? (21)]; *3*) adult eupneic breathing [Do they provide a tonic drive? Are they an expiratory oscillator? (31)]; *4*) the integration of carotid body and hypothalamic inputs. Further it is unclear whether all these functions are unique to RTN Phox2b-immunoreactive (ir) cells or involve other RTN neuronal types. Examination of a neuronal marker shows substantial numbers of non-Phox2b cells in the RTN (81), and in earlier studies we have shown dramatic effects on the CO_2 response following kainic acid lesions (a toxin affecting all cells with glutamate receptors) in decerebrate cats (61) and in awake rats (2). We have little doubt that the RTN is a vital structure and that it receives multiple inputs; but is it solely responsible for CCR in adults? Even cell type-specific dysfunction of RTN neurons may not answer this question.

The Richerson laboratory has portrayed the serotonin (5-HT) neurons of the MR as the primary CRR detector cells (8, 67–69, 82). By recording responses of these cells grown in culture as well as in brain stem slices, his group has found them to be intrinsically responsive and sensitive to CO_2/pH. Studies of the function of MR 5-HT neurons in vivo that utilize lesions, inhibition, stimulation, or genetic disruption indicate a functional role for 5-HT neurons in CCR (5, 13, 30, 59) but suffer from the same concerns as stated above for similar studies of RTN neurons as MR 5-HT neurons also have many other functions in addition to being putative CO_2/pH detectors. Given the evidence that MR 5-HT neurons are CO_2 sensitive in vitro and function in the CCR response in vivo, one is puzzled by the rescue of the decreased CO_2 response in the Lmx1b knockout mouse, which is absent of all 5-HT neurons, by administration of 5-HT (30). If the 5-HT neurons function as primary CO_2/pH detectors then how can the response be rescued by administration of 5-HT? One answer may be related to our findings with focal acidification of the rostral MR. Ventilation was increased only in sleep while the lesions, inhibition, and genetic studies were performed in wakefulness. Does the CO_2 sensitivity of MR 5-HT neurons function most importantly in sleep? Another possible explanation is that the added 5-HT "rescues" the response by enhancing the effectiveness of other CCR sites and that of the carotid body as demonstrated in our study of simultaneous focal acidification of RTN and rostral MR (13). MR 5-HT neurons could be important detectors in some conditions and enhancers in other conditions.

In fact, many brain stem neurons when studied in isolation (with and without synaptic input) can be excited (or inhibited) by CO_2/pH (18). And inhibition and lesions of other putative CCR sites, e.g., the LC (6, 32, 63) or NK1R-ir cells in the RTN (58) and ventral medulla (53), can also disrupt the CO_2 response.

SUMMARY AND FUTURE DIRECTIONS

As genetic and developmental studies provide new information regarding the specific phenotypes of different neurons within the various putative CCR areas, then new strategies can be employed in vivo to test the function of highly specified neuronal and glia types, e.g., viral transfection, opticogenetics, transgenic. Of special utility would be the identification of a cell type-specific CO_2/pH detection molecule. Still, in our view, the proof of physiological function and importance lies within studies performed in conscious animals during sleep and wakefulness. Whether or not our view of system organization, as shown in an early form in Figs. 1 and 2, prevails, there is little doubt that the putative CCR sites interact in significant ways for system function when studied in the intact, unanesthetized animal and these interactions differ in sleep and wakefulness. It is discovering how nature uses and manipulates the intrinsic properties of cells, here with regard to CO_2/pH sensitivity, which yields the ultimate satisfaction of system understanding.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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