

Neuronal Connections of a Ventral Brainstem Respiratory Chemosensitive Area

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I. Introduction

Several investigations have implicated neurons at the ventral brainstem in the central chemical regulation of breathing (1–3). This chemosensitive region lies ventral to the inferior olives at the level where the hypoglossal rootlets exit the brainstem and may be reached by fluids superfusing the ventrolateral medullary surface (VMS) (Fig. 1).

Electrical stimulation of the region of maximal chemosensitivity (area L) in the cat resulted in a marked increase in ventilation (2,4). Berndt et al. (5) demonstrated that superfusion of this area with 2% procaine solution in peripheral chemoreceptor-denervated animals completely abolished the ventilatory response to electrical stimulation, causing respiratory arrest. However, electrical stimulation of the respiratory center in these animals still elicited an inspiratory response. It appears, therefore, that these superficial structures might be essential in driving the respiratory center in anesthetized cats in the absence of peripheral afferents.

If it is assumed that specialized receptors on the ventrolateral surface of the medulla oblongata are capable of sensing changes in their chemical environ-

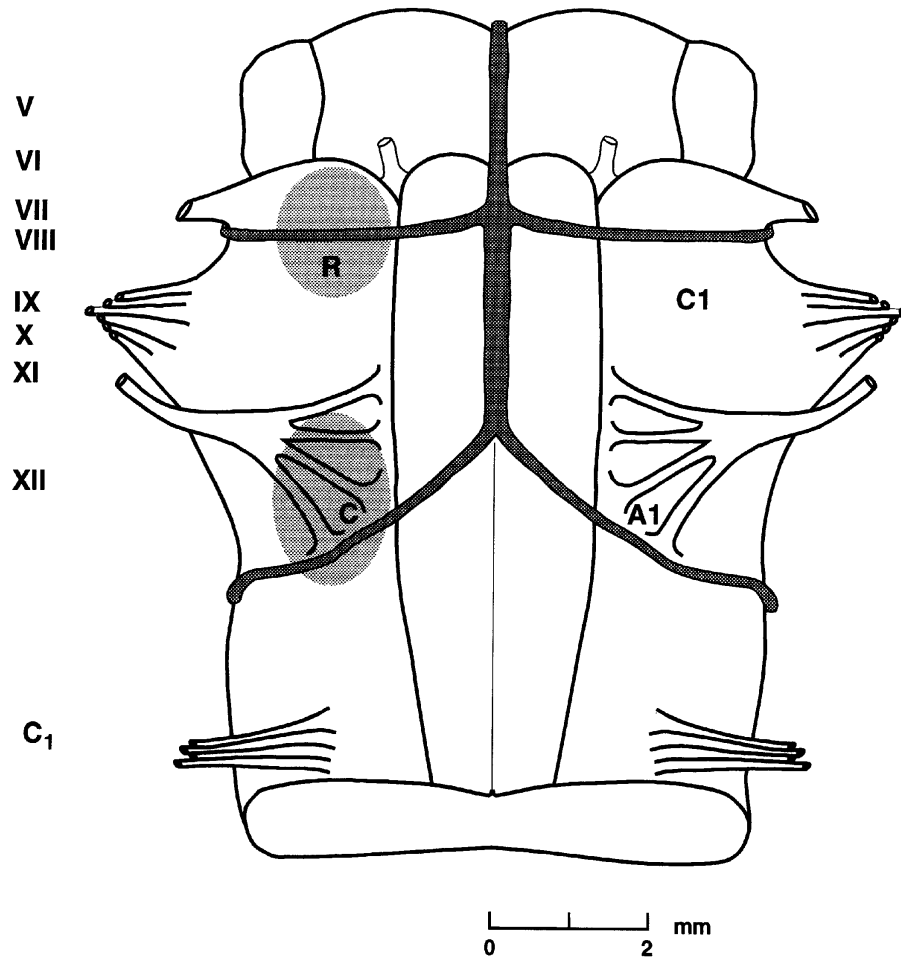


Figure 1 Ventral surface of the medulla oblongata of the rat. On the left half of the diagram, two chemosensory regions are indicated by the symbols R and C, representing the rostral and caudal chemosensitive zones, respectively. V–XII = cranial nerves.

ment, then these receptors should respond by transmitting information to the respiratory centers, thus providing the centers with the necessary input required for reflex adjustments of pulmonary ventilation.

C-fos and other immediate-early genes, in response to various physiological and pharmacological stimuli, induce concomitant nuclear binding proteins that appear to play a role in signal transduction and to function as intracellular third messengers. Sato et al. (6) described the distribution of the *c-fos* in cells of the ventral medulla oblongata that were responsive to 15% inspired CO_2 . Immunocytochemical evaluation of the tissue revealed *c-fos*-positive neurons that were located within 150 μm of the VMS in the caudal ventrolateral medulla oblongata (cVLM); with a majority of cells within the first 50 μm .

In the present study, we have evaluated the neuronal connections of the caudal chemosensitive area with other respiratory control regions in the brainstem using horseradish peroxidase (HRP)-tetramethyl benzidine (TMB) reaction product as a marker for neuronal projections.

Seven rats weighing 300 to 400 g, anesthetized with chloralose urethane (200 mg/kg glucochloralose and 400 mg/kg urethane) were used. The muscoli longi capitis and recti capitis ventrales, as well as the pars basalis os occipitale, the ventral portion of the atlas, and the processus odontoideus of the epistropheus were removed. The dura was then opened and reflected laterally to expose the ventral surface of the medulla oblongata. Rectal temperature was maintained constant at 37°C. The animals were then placed in the supine position with the head stabilized in a stereotaxic apparatus.

Following surgical exposure of the ventral brainstem surface, a solution of 0.02 to 0.05 μL of 4% HRP (Sigma VI) was injected into the caudal chemosensitive area on the VMS with the aid of a micromanipulator in a stereotaxic coordinate system. A 1- μL Hamilton syringe was used. At 48 h postinjection of HRP, the head of the animal was perfused and 40 μm tissue sections were processed for the TMB reaction product according to the protocol of Mesulam (7). In another series of experiments, wherever anterograde labeling was noted in the present study, microinjections of HRP were made to validate the presence of projections to that site, for example the nucleus ambiguus (NA).

In Fig. 2, the injection site was located in the cVLM overlying the exit of the hypoglossal (XII) rootlets. Only those cases where the injection was confined to the VMS were chosen for this report. A small ipsilateral projection appears to exist between the NA [or ventral respiratory group (VRG)] and the VMS. Light deposits of HRP reaction product could also be visualized in a few small, oval neurons of the ventrolateral nucleus tractus solitarius (NTS)—that is, the dorsal respiratory group (DRG), bilaterally.

Ipsilateral to the cVMS injection site, heavy labeling with HRP reaction product occurred in the nerve XII fibers traversing the medulla and in the large, multipolar neurons of the hypoglossal nucleus. This may represent uptake of HRP from the surrounding medium by nerve XII fibers. Occasional large, multipolar neurons containing HRP were also noted within the medullary reticular nucleus in the vicinity of the fibers of XII and in the lateral reticular nucleus (LRtN) (Fig. 3).

Contralateral to the injection site, HRP reaction product was detected in a few unique areas; the parvocellular region of the lateral reticular nucleus (LRt-PC), the ventrolateral and dorsolateral aspects of the inferior olivary nucleus (IO) and along the VMS. Small to medium sized neurons located in the contralateral medial and ventrolateral IO as well as superficial neurons within the thickened marginal glia (TMG) were variably labeled with HRP. A few large multipolar neurons within the contralateral LRt-PC were heavily retrogradely labeled with HRP and fibers emanating from their somata would be visualized directed toward the injection site (Fig. 4). Mesulam (7) and Kahlia

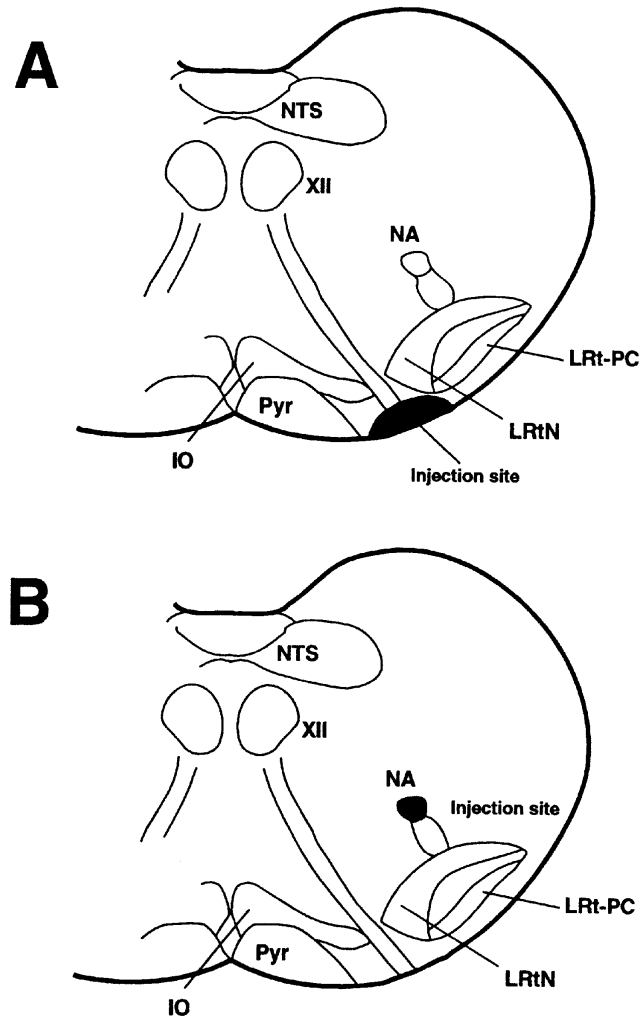


Figure 2 Diagrammatic representation of the location of HRP injection sites in the rat caudal medulla oblongata. (A) Injection site located at the VMS in the region of the hypoglossal rootlets. (B) Injection site located within the NA; one of the sites of anterograde labeling from the VMS. NTS = nucleus tractus solitarius; XII = hypoglossal nucleus; NA = nucleus ambiguus; LRT-PC = parvocellular region of the lateral reticular nucleus; LRTN = lateral reticular nucleus; Pyr = pyramidal tract; IO = inferior olivary nucleus.

and Wells (8) have demonstrated the usefulness of using TMB as the substrate for HRP histochemistry in studies where the anterograde transport of HRP is desirable. In these studies, a few tortuous fibers and axon terminal fields containing anterogradely labeled HRP could be detected within the NA, LRT-PC, and along the VMS, indicating that reciprocal connections exist between VMS,

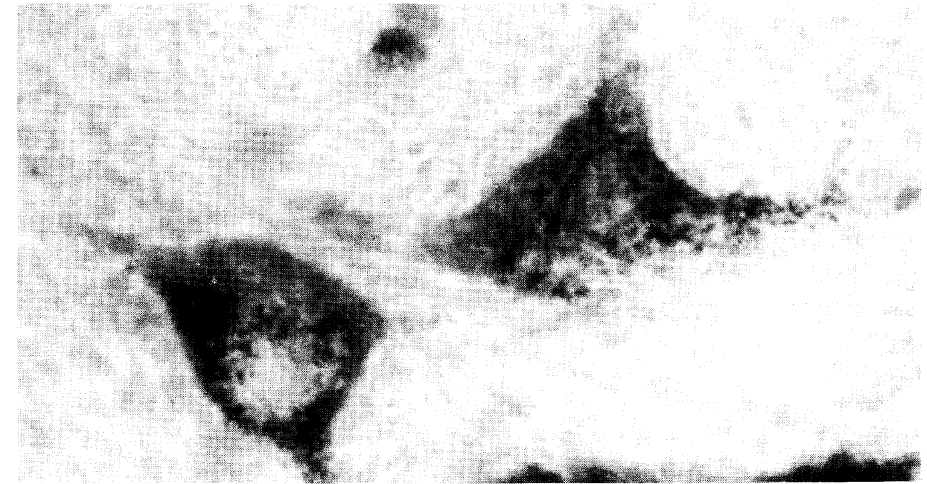


Figure 3 Light micrograph of a pair of HRP-positive neurons in the medullary reticular nucleus retrogradely labelled from a VMS injection site $\times 40$. Neutral Red counterstain.

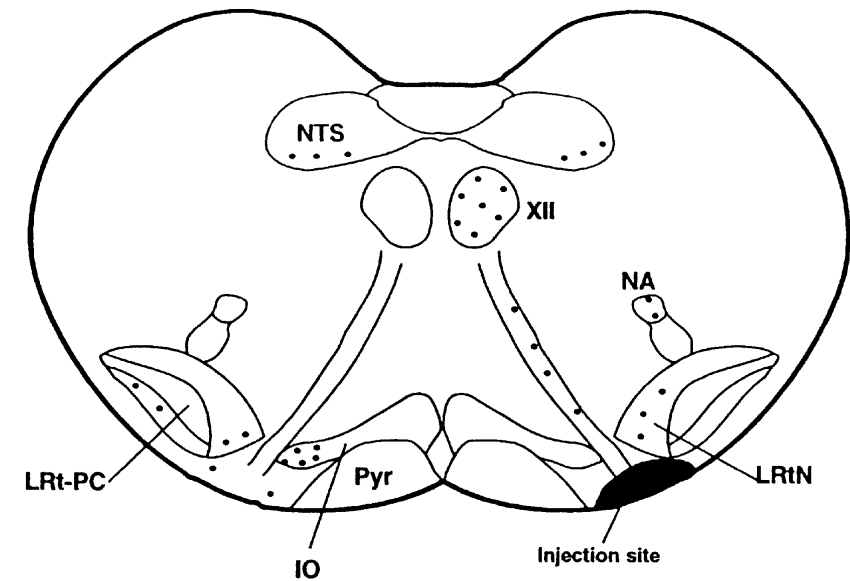


Figure 4 Diagrammatic representation of projections from various medullary subnuclei to the cVMS. Each dot represents a neuron retrogradely labeled with HRP from a VMS injection site. Ipsilateral retrograde HRP labeling was visualized in the LRTN, NA, NTS, and nerve and nucleus of XII. Contralateral retrograde HRP labeling was noted in the NTS, LRT-PC, IO, and along the VMS. LRTN = lateral reticular nucleus, NA = nucleus ambiguus, NTS = nucleus tractus solitarius, XII = hypoglossal nucleus, IO = inferior olivary nucleus, LRT-PC = parvocellular region of the lateral reticular nucleus, Pyr = pyramidal tract, and VMS = ventral medullary surface.

ambiguous, and LRt-PC neurons. Verification studies were subsequently performed by microinjection of HRP into sites demonstrating anterograde labeling. These observations agree with the reports of Norgren (9), who, with the use of tritiated proline or leucine, described projections emanating from the NA that project to the NTS. Indeed, Gallagher and Pert (10) and Walberg (11) have found that extensive reciprocal connections exist between reticular nuclei. Neurovascular elements, variably labeled with HRP, were noted in superficial regions of the medulla. These cells that are in close contact with blood vessel walls are reminiscent of the neurovascular elements described by Scheibel and Scheibel (12) and Scheibel et al. (13).

II. Summary

Regions of respiratory chemosensitivity that respond to cerebrospinal fluid pH changes and inspired carbon dioxide have been described on the VMS. In this investigation, HRP, a neuronal tracer, was utilized to ascertain the possible connections between the VMS and the deeper-lying respiratory structures. In spontaneously breathing, chloralose-urethane anesthetized rats, 0.02 to 0.05 μL of 4% HRP was microinjected into the cVMS via a 1- μL Hamilton syringe mounted on a stereotaxic coordinate system. The animals were maintained for a minimum period of 48 h to allow for the axonal transport of HRP. The injection site was located over the hypoglossal rootlets and less than 500 μm into the caudal VMS. The results reveal that the majority of neuronal connections with the caudal VMS arise (retrogradely) from (1) Ipsilaterally: lateral reticular nucleus, the nucleus ambiguus, and the nucleus tractus solitarius. HRP reaction product was also visualized within the nerve fibers and large multipolar neurons of the hypoglossal nucleus. (2) Contralaterally: IO nuclei and the parvocellular region of the LRtN (with a few neurons that have extremely long processes directed towards the midline). Neuronal connections were also identified by anterograde labeling, which indicated the presence of projections arising from the cVMS that may impinge on the respiratory centers. This investigation demonstrates that (a) superficial neuronal elements project to deeper-lying respiratory structures such as the NA and NTS; (b) bilateral connections exist between the LRt-PC nuclei; (c) possible bilateral connections between lateral inferior olivary nuclei and VMS cells, and (d) neurovascular elements may play a role in respiratory control mechanisms within the ventrolateral medulla oblongata. It is therefore possible that the lateral horns of the IO, the parvocellular regions of the LRtN, and superficial neurons within the TMG are involved in higher-order processing within the central respiratory chemoreceptor reflex pathway.

Acknowledgments

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Neurotransmitters in Cardiorespiratory Regulation at the Ventral Surface of the Medulla Oblongata

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I. Introduction

It is well recognized that ventilatory response to CO₂ persists after denervation of arterial chemoreceptors. Pioneering work of Hans Loeschcke and his co-workers led to the identification of the ventral surface of the medulla oblongata (VMS) as an important neural substrate for mediating ventilatory response to CO₂. Since then, attention of respiratory physiologists has been focused on the VMS in elucidating its role in ventilatory homeostasis. Later studies by Feldberg, Guertzenstein and their coworkers led to the notion that VMS is also an important substrate for maintaining circulatory homeostasis. The purpose of this article is to review briefly the role of neurochemicals in the regulation of respiration and blood pressure by neural substrates in the VMS.

II. Identification of Neural Substrates in the VMS That Influence Breathing

It is well established that respiratory responses can be elicited only from certain regions of the VMS often called "chemosensitive areas." The following is a

brief description of the studies that led to the identification of different areas on ventral brain stem that influence breathing.

It has long been thought that changes in blood gases, especially CO₂, affect breathing by their direct action on central neurons controlling respiration (1). In 1952, von Euler and Soderberg (2) suggested that the neural substrate responding to CO₂ is distinct from the respiratory-related neurons. Based on experiments with intraventricular perfusion of the brain, Leusen (3) suggested that chemosensitive neurons might be located superficially in the brain stem. In 1958 Loeschcke and Koepchen (4) concluded that neural substrates responding to changes in pH of the extracellular fluid (ECF) should be in close proximity to the VMS. Subsequently Mitchell, Loeschcke, and coworkers (5,6) showed that application of local anesthetics or cold mock cerebrospinal fluid (CSF) to the VMS depresses ventilation, but not when they are applied directly to the floor of the fourth ventricle. The studies of Mitchell, Loeschcke, and Schläfke have led to the identification of three areas in ventral surface of brainstem that affect breathing (6). They are located in rostral, intermediate, and caudal parts of the VMS. Electrical stimulation, application of chemicals and pledgets soaked in acidic mock-CSF to the rostral and caudal areas all increase respiration (6). In peripherally chemodenervated animals, bilateral cold blockade of intermediate areas led to apnea and attenuates or abolishes CO₂-induced increases in breathing. These studies led to the notion that the rostral and caudal areas contain neuronal substrate(s) for sensing the changes in CO₂ (or, more appropriately, changes in ECF pH); whereas the intermediate area is necessary for integration of the inputs from chemosensitive areas (6).

III. Influence of Neurochemicals on Breathing

Like other parts of the nervous system, the VMS contains several classes of neurochemicals. These include biogenic amines, amino acids, and neuropeptides. Several studies have examined their influence on ventilation. Following is a brief review of the effects of different chemicals on breathing when they are applied topically or microinjected into the chemosensitive areas of VMS.

Mitchell, Loeschcke, and collaborators (5) reported increases in respiration following local application of acetylcholine (ACh) or nicotine to the VMS. These studies were further extended by Dev and Loeschcke (7), who examined the effects of ACh on respiration in detail. These authors demonstrated that the stimulatory actions of ACh were confined to those areas of the VMS that correspond to rostral and caudal chemosensitive areas. More importantly, topical application of atropine, a muscarinic receptor antagonist to chemosensitive areas, markedly attenuated ventilatory response to CO₂. Furthermore, physostigmine applied to chemosensitive areas increased respiration (7). Other biogenic amines—such as norepinephrine, epinephrine, or serotonin—had no effect on respiration when applied to chemosensitive areas (7). From these results,

it was suggested that transmission of a CO₂-induced ventilatory response requires cholinergic mechanism(s) (8). Haxhiu, Cherniack, and their collaborators (9) have shown that cholinergic agonists applied to the VMS increase the phrenic and respiratory discharge of the hypoglossal nerve. However, the effects of cholinergic agonists were greater on upper airway muscles than on phrenic nerve activity. These studies suggest that cholinergic agonists acting on chemosensitive areas of the VMS affect the output of the respiratory nerves differentially. Such differential effects could be of physiological significance in coordinating the respiratory behavior of pumping versus upper airway dilating muscles.

There is considerable evidence that amino acids affect breathing when applied to the VMS (10). In general, amino acids such as gamma-aminobutyric acid (GABA), its agonist, muscimol, taurine, and glycine inhibit breathing and depress ventilatory response to CO₂. On the other hand, application of glutamate or its agonist, *N*-methyl-D-aspartate (NMDA), stimulates breathing. When applied to the VMS, amino acids affect not only breathing but also the tone of the tracheal smooth muscle and airway secretion (11). These studies indicate that amino acids, by acting on the neurons of the VMS, can potentially influence not only pumping muscles of respiration but also upper airways.

In recent years several neuropeptides have been identified in the ventral brainstem that may function as neurotransmitters or modulators. These include tachykinin peptides, notably substance P (SP), enkephalins, somatostatin, NPY, neurotensin, and atrial natriuretic peptide (ANF) (Ref. 10 and Kumar, this volume). As elsewhere in the nervous system, some of these peptides are colocalized with other chemicals. For example, SP is colocalized with serotonin in the neurons of the rostral ventrolateral medulla (RVLM). Furthermore, Kumar and coworkers have shown that the VMS contains the enzyme neutral endopeptidase (NEP), which hydrolyzes many of the neuropeptides. Topical application of tachykinins like SP and neurokinin A (NKA) to chemosensitive areas increases phrenic nerve activity as well as the tone of the tracheal smooth muscle. Topical application of phosphoramidon, an inhibitor of NEP, potentiates respiratory response to CO₂ (12). Neurotensin also stimulates phrenic nerve activity and increases tracheal tone. The effects of neurotensin on tracheal smooth muscle appear to be mediated via parasympathetic nerves innervating the tracheal smooth muscle (13). On the other hand, peptides like somatostatin and ANF inhibit phrenic nerve activity and decrease tracheal tone (14). Interestingly, at certain doses, ANF causes a profound decrease in tracheal tone but no effect on efferent phrenic nerve activity (see Fig. 4 of Ref. 14). Thus, these studies indicate that tachykinins and neurotensin are stimulatory whereas somatostatin and ANF are inhibitory to respiration when applied to chemosensory regions of the VMS.

Thus far, studies described above demonstrate that, when applied to the VMS, neurochemicals cause pronounced changes in respiration. It is noteworthy

that certain neurochemicals at given doses affect upper airway muscles more than efferent phrenic nerve activity. Likewise, pronounced changes are seen in tracheal tone without any noticeable effects on efferent phrenic nerve activity. Thus, any conclusions to be drawn about the effects of neurochemicals on respiration must take into account the heterogeneity of responses of different respiratory muscles. While the differential responses in respiratory muscles may have important physiological implications, they might also represent the different thresholds and anatomical connections from chemosensitive neurons to respiratory motoneurons.

IV. Identification of Neural Substrates in the VMS Influencing Blood Pressure

In 1972, Feldberg and Guertzenstein (15) reported that topical application of chemicals to the VMS leads to marked changes in arterial blood pressure. These authors noted that cardiovascular alterations could be elicited from two areas in the VMS that correspond closely to the caudal and rostral part of intermediate chemosensitive areas. Bilateral application of glycine or bilateral lesioning of the rostral area causes a profound fall in blood pressure (16). Application of nicotine, physostigmine, or carbachol to the caudal area also produces a fall in blood pressure (16). Later studies have shown two collections of neurons that contain catecholamines. Stimulation of these neurons results in strong vasomotor responses. One group, located more caudally, contains norepinephrine; these are often referred to as A_1 neurons (17,18). Stimulation of these neurons produces a fall in blood pressure. Some but not all A_1 neurons project to the hypothalamus and influence the secretion of arginine vasopressin (AVP). However, the A_1 group has no direct connection to the spinal cord. A second group of neurons is located more rostrally and contains epinephrine; these are called the C_1 group (17,18). Blockade of C_1 neurons by tetrodotoxin prevents the vasodepressor effects caused by the stimulation of A_1 neurons. A direct anatomical connection of C_1 neurons to the intermediolateral column of the spinal cord has been reported (19,20). These neurons constitute vasomotor presympathetic neurons (VPN) and contain epinephrine and probably also glutaminergic cells (21). Stimulation of the rostral group of neurons raises blood pressure by producing vasoconstriction. Ablation of the rostral group of neurons lowers blood pressure and impairs the reflex response to baroreceptor stimulation (17-20). These findings have led to the notion that neurons in the RVLM provide tonic sympathetic vasomotor tone for maintaining blood pressure and that they integrate a wide variety of vasomotor reflexes (17-20).

V. Influence of Neurochemicals on Blood Pressure

Local application of carbachol or physostigmine into the RVLM produces fall in blood pressure that can be prevented by atropine. These findings led to the

suggestion that cholinergic mechanisms exert a tonic inhibitory influence on RVLM neurons (22). It is well known that administration of clonidine decreases blood pressure. The hypotensive effects of clonidine were attributed to its actions on neurons in RVLM. The effects of clonidine in the VMS are mediated either by alpha-2 adrenergic receptors (see Guyenet et al., this volume) or by imidazoline receptors (see Ernsberger et al., this volume), or both. Application of epinephrine to the RVLM inhibits vasomotor activity, probably by acting on alpha-2 adrenergic receptors (23).

Microinjection of glutamate into C_1 neurons produces marked increases in blood pressure (10). Application of the inhibitory amino acid GABA to C_1 neurons lowers blood pressure. Similar injection of GABA into A_1 neurons, however, raises blood pressure (24).

Neurons in the RVLM region contain neuropeptides such as SP and neuropeptide Y (10). It has been shown that SP mediates some of the pressor effects elicited by stimulation of neurons in the RVLM (10). Application of somatostatin or ANF in the vicinity of the RVLM neurons produced a small but significant fall in blood pressure (14).

VI. Summary and Perspectives

There is little doubt that neural substrates in the VMS play important roles in the regulation of breathing and circulation. The last few decades have witnessed considerable progress in the distribution and characterization of the effects of neurochemicals on respiration and blood pressure. Several questions, however, remain unanswered. First, are there separate neurons that regulate respiration and blood pressure? Obviously, the answer to this question cannot be a simple one. Several lines of evidence, however, indicate that neurons mediating respiratory and vasomotor responses are closely intermingled and interconnected and may not be identical.

Although the effect of neurochemicals on respiration and ventilatory responses to CO_2 has been well documented, little is known regarding the contribution of these chemicals to the transmission of the CO_2 signal. Also, no information is available as to the release of neurochemicals from the chemosensitive areas of the VMS in response to graded changes in CO_2 . Similar information will also be of value in clarifying the role of neurochemicals of the VMS region in the regulation of blood pressure. Furthermore, little attention has been given to the question of whether or not the effects of these chemicals on respiration and blood pressure are secondary to changes in cerebral blood flow.

In other areas of the nervous system, there is a good deal of crosstalk among various neurotransmitters and modulators. For instance, neuropeptides have been shown to influence the release of other biogenic amines, such as acetylcholine and catecholamines. Whether such interactions among these molecules

exist in the VMS is yet to be investigated. Recent studies from Truth and Neubauer and their coinvestigators (this volume) indicate that carbonic anhydrase-containing neurons in the VMS may subservise the function of CO₂ sensing. If this is further confirmed, then it would be important to characterize the distribution of neurochemicals in this subset of carbonic anhydrase-containing VMS neurons and to assess their possible anatomical connections to respiratory and cardiovascular motoneurons.

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