Review

Differential regulation of the central neural cardiorespiratory system by metabotropic neurotransmitters

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Central neurons in the brainstem and spinal cord are essential for the maintenance of sympathetic tone, the integration of responses to the activation of reflexes and central commands, and the generation of an appropriate respiratory motor output. Here, we will discuss work that aims to understand the role that metabotropic neurotransmitter systems play in central cardiorespiratory mechanisms. It is well known that blockade of glutamatergic, gamma-aminobutyric acidergic and glycinergic pathways causes major or even complete disruption of cardiorespiratory systems, whereas antagonism of other neurotransmitter systems barely affects circulation or ventilation. Despite the lack of an 'all-or-none' role for metabotropic neurotransmitters, they are nevertheless significant in modulating the effects of central command and peripheral adaptive reflexes. Finally, we propose that a likely explanation for the plethora of neurotransmitters and their receptors on cardiorespiratory neurons is to enable differential regulation of outputs in response to reflex inputs, while at the same time maintaining a tonic level of sympathetic activity that supports those organs that significantly autoregulate their blood supply, such as the heart, brain, retina and kidney. Such an explanation of the data now available enables the generation of many new testable hypotheses.

Keywords: cardiorespiratory integration; baroreflex; somatosympathetic; chemoreflex; peptide

1. INTRODUCTION

Sympathetic nerve activity is crucial for the regulation of many bodily functions including maintenance of arterial blood pressure, renal and reproductive function and vision. Despite decades of investigation, key questions about central cardiorespiratory regulation remain poorly understood and unexplored.

Figure 1a illustrates some pathways in the brainstem that can regulate the central control of the cardiorespiratory system. At the most simplistic level, central cardiovascular control is concerned with the maintenance of 'tone' in the cardiovascular system and the elaboration of reflex responses to sudden changes in blood pressure, oxygen, pH or other inputs such as regional requirements for increased oxygenation. Similarly, the same brainstem areas that regulate the cardiovascular system also contain neurons that generate a normal respiratory activity and are responsible for muscle tone in the upper airways, and for swallowing. The colocation of these functions could well be a consequence of their appearance in evolution rather than as a requirement for the control of the two systems. However, the colocation of all these vital systems, and their common blood supply, does mean that structural lesions in this region are commonly massively debilitating or fatal (Telerman-Toppet *et al.* 1982). Although our knowledge of the functional neuroanatomy of the ventrolateral medulla, which for the purposes of our discussion extends from the facial nucleus to the spinal cord is considerable, our knowledge of how the different groups of neurons form precise connections with other groups is still uncertain. Many of the 'big' questions that face cardiorespiratory neuroscientists today are the same as those that were prominent over the past decades. These intractable questions include:

(i) How is blood pressure maintained at a mean of approximately 100 mm Hg so as to enable adequate perfusion of all organs?
(ii) What role do individual brainstem and spinal cord neurons play in the maintenance of blood pressure?
(iii) Why do so many of the important central neurons express so many different neurotransmitters and receptors?

These questions are general, but their specific corollaries are no more tractable, and include:

(i) Why do some sympathoexcitatory neurons in the rostroventrolateral medulla oblongata
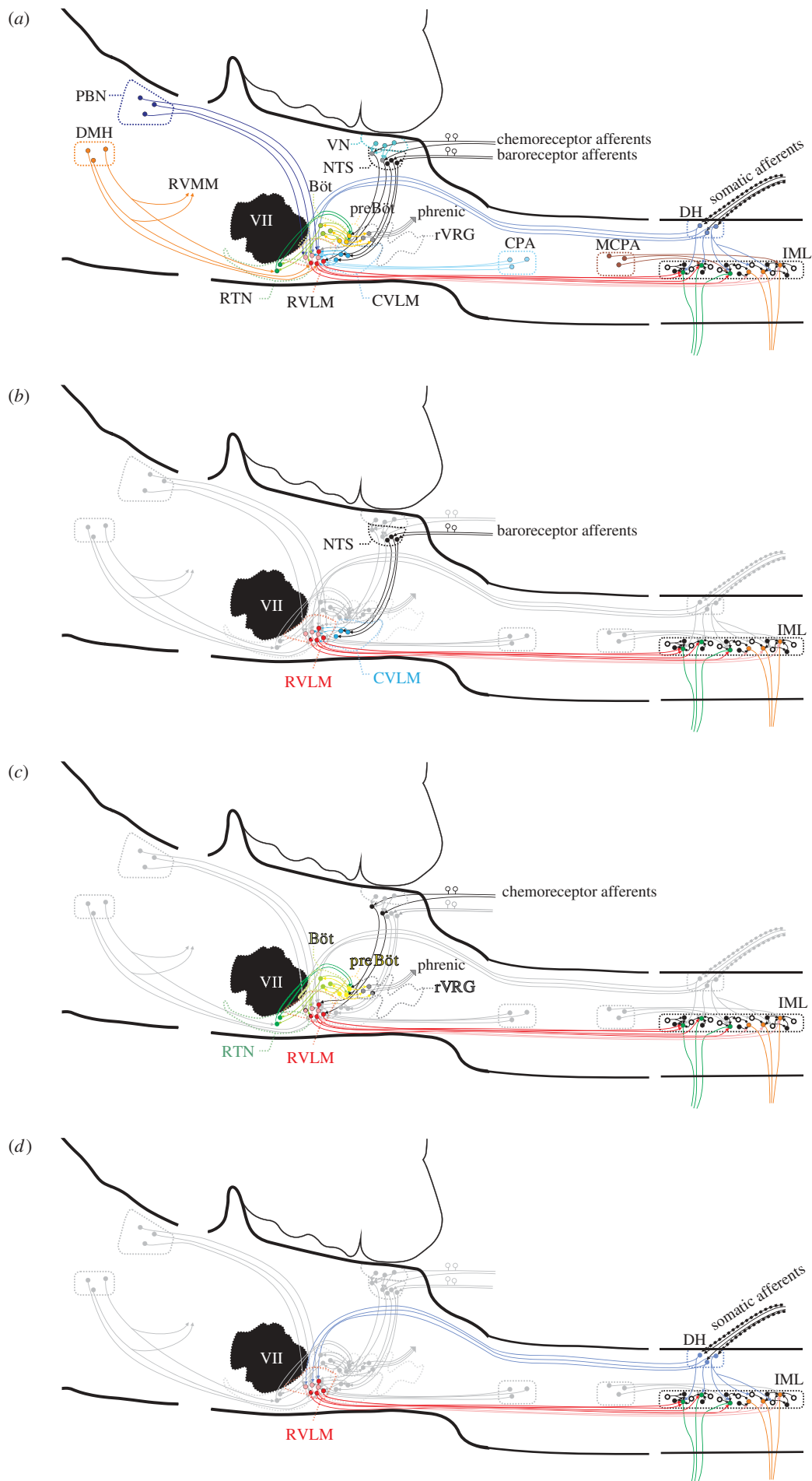
Figure 1. *Caption opposite.*

Figure 1. (*Opposite.*) A diagram of pathways in the regulation of the cardiorespiratory system: (*a*) all pathways overlapped. The bulbospinal red pathways are in the RVLM (figure 2*a*) and integrate information from the centre and the periphery. The output from this nucleus is crucial for maintaining normal sympathetic tone. PBN, parabrachial nucleus; DMH, dorsomedial hypothalamus; CVLM, caudal ventrolateral medulla; VLM, ventrolateral medulla; rVRG, rostral ventral respiratory group; CPA, caudal pressor area; MCPA, medullo cervical pressor area; IML, intermediolateral cell column; RVMM, rostral ventro-medial medulla; VII, facial nucleus; RTN, retrotrapezoid nucleus; preBöt, preBötzinger neurons; VN, vestibular nucleus. (*b*) The baroreflex pathway is shown on its own. Stretch receptor afferent neurons from the aortic arch and carotid sinus and the neurons synapse in the nucleus tractus solitarius (NTS). Neurons in the NTS then activate inhibitory neurons (blue) in the caudal ventrolateral medulla, which in turn inhibit the neurons in the RVLM; this intense gamma-aminobutyric acid (GABA)-mediated inhibition inhibits sympathetic outflow, causing blood pressure and sympathetic nerve activity to fall. Note also the yellow respiratory neurons that modulate the activity of the cardiovascular neurons (also in *c*). (*c*) The pathways for peripheral and central chemoreceptors are shown. Central chemoreceptors are highly responsive to changes in CO_2 and are found in the retrotrapezoid nucleus. Many of these chemosensitive neurons (greater than 40%) are galaninergic and Phox2b positive, but all lack tyrosine hydroxylase (Stornetta *et al.* 2009; figure 2*b*). Peripheral chemoreception emanates from the car-otid body. Neurons terminate in the medial NTS (like the baroreceptors). From here, the excitatory information passes to both respiratory and cardiovascular neurons. (*d*) The somatosympathetic pathway is shown in an abbreviated form. Afferent nociceptive pathways enter the spinal cord in the dorsal roots, activate circuits locally, and at several stations throughout the neuraxis including the RVLM. This pathway is excitatory and results in the appearance of a variable number of peaks in sympathetic nerve activity, depending on which nerve is recorded from. In the case of the greater splanchnic nerve, this is generally two peaks.

(RVLM) appear to have the capacity to syn-thesize adrenaline (Hökfelt *et al.* 1974; Phillips *et al.* 2001) and substance P (Pilowsky *et al.* 1986*a*,*b*; Milner *et al.* 1988*a*,*b*; Li *et al.* 2005), as well as many other neurotransmitters, in addition to glutamate?

(ii) Which brainstem neurons regulate which motor outputs?

(iii) Does the 'chemical coding' seen in different cardiorespiratory neurons correspond to a 'functional fingerprint'?

The pot of gold at the end of this rainbow is a much better understanding of how the autonomic nervous system is regulated and consequently, the development of a deeper and more complete understanding of the pathophysiology of the underlying disorders of auto-nomic control, that range from hypertension to asthma, and cost our community considerable social and material capital.

First, it is necessary to take a step back to describe the system that we are investigating and then to select a manageable number of examples with which to develop the concepts discussed earlier. What is known about the central circuitry responsible for the maintenance of arterial blood pressure and the various reflex inputs that affect it? Normally, there is a continuous flow of excitation from the sympathetic nervous system to the periphery in order to maintain a basal level of tone to blood vessels, a certain amount of release of adreno-medullary hormones (adrenaline and noradrenaline) and a heart rate that can be varied up and down according to circumstance. Ventilation, on the other hand, is a discontinuous activity in the sense that the peripheral organs regu-lated by central respiratory generators stop their output entirely between each breath and, in certain circumstances, may not provide any output at all.

Figure 1*a*–*d* illustrates the neurons responsible for maintaining arterial blood pressure. The key thing to note is that a small nucleus in the rostral part of the RVLM, a tubular structure 0.6 mm in length and 0.2 mm in diameter in rats, defines its greater part. This nucleus is important because any intervention that decreases or eliminates its normal function causes sympathetic activity to fall to zero (acutely at least), all sympathetic reflex activity to be eliminated and blood pressure to fall to a level similar to that seen after high spinal cord transection (Schreihofer *et al.* 2005; Braga *et al.* 2007). This is the case in all vertebrates examined to date, from man (Telerman-Toppet *et al.* 1982) to rat (Suzuki *et al.* 1994; Pilowsky & Goodchild 2002; Miyawaki *et al.* 2003). Chronically, the contribution of the sympathetic ner-vous system in awake animals remains controversial, with some reports favouring an almost completely endocrine (angiotensin II, vasopressin) basis for the restoration of pressure, while others suggest a sym-pathetic component as well. Some studies favouring a role for sympathetic nerves in maintaining sympath-etic nerve activity after spinal cord transection are based on pharmacological interventions with α and β adrenergic blockade. Modern studies suggest that sympathetic nerves are not active following acute or chronic cervical spinal cord transection (Trostel & Osborn 1994).

It has been clearly demonstrated that the RVLM is crucial for the maintenance of sympathetic tone and elaboration of reflex responses; both tone and reflex control are lost after acute destruction of the RVLM. On the other hand, there is little effect on arterial blood pressure or sympathetic nerve activity after the destruction of any other area unless the RVLM is also inactivated. Despite this, chemical inactivation of the RVLM, with the resultant immediate fall in sympathetic tone, blood pressure and abrogation of reflexes, does not eliminate the persistent potent hypertensive and sympathoexcitatory effects that can be elicited from other sites such as the medullo-cervical pressor area (Seyedabadi *et al.* 2006). Many other sites in the brain apart from the RVLM have direct or indirect inputs to sympathetic preganglionic neurons, but their activity, if any, appears insufficient to sustain any apparent sympathetic activity in the absence of the RVLM. A5 neurons, for example, have a direct spinal projection and are likely to innerv-ate sympathetic preganglionic neurons (SPN); however, electrophysiological studies suggest that A5

neurons lack a cardiac-related rhythm in their firing pattern (Byrum *et al.* 1984). In fact, demonstrating a monosynaptic connection between an individual supraspinal neuron and an individual SPN has proved to be a very difficult problem. To date, only small numbers of barosensitive connections between the RVLM and the spinal cord have been revealed electrophysiologically (McAllen *et al.* 1994; Oshima *et al.* 2006, 2008). Similarly, the numbers of synapses between C1 neurons and SPN appear to be very small (Llewellyn-Smith *et al.* 1991). Figure 1*b–d* illustrates the sympathetic baro-, chemo- and somatosympathetic reflexes, respectively. The RVLM neurons and the inhibitory neurons in the caudal ventrolateral medulla also receive inputs that cause sympathetic activity to burst in phase with phrenic nerve discharge (inspiration; Haselton & Guyenet 1989; Miyawaki *et al.* 1995; Mandel & Schreihofer 2006).

2. HISTORICAL ASPECTS

Early studies, in the late nineteenth century, identified the ventral brainstem as an area crucial for the tonic and reflex regulation of the cardiovascular system (Fye 1986; Seller 1996). Subsequently our understanding of the different compartments of the ventral brainstem has become more and more refined (Pilowsky & Goodchild 2002) as techniques such as drug microinjection (Goodchild *et al.* 1982; Lipski *et al.* 1988; Monnier *et al.* 2003) and electrophysiology combined with dye labelling, immunohistochemistry and tract tracing were applied to develop our understanding of these regions (Pilowsky *et al.* 1994*b*; Sun *et al.* 1994, 1995, 1997). The neurochemical and receptor content of barosensitive bulbospinal neurons in the RVLM has been the subject of intensive investigation over the past 30 years since the first report by Hökfelt and colleagues in 1974 (Hökfelt *et al.* 1974) that a population of neurons existed in the RVLM (figure 2) that contained the enzyme phenylethanolamine-*N*-methyltransferase (PNMT), which is the key (but not rate limiting) enzyme in the biosynthetic pathway for adrenaline. Subsequent studies combining immunohistochemistry, PCR and *in situ* hybridization revealed that many bulbospinal neurons in the RVLM contained all of the biosynthetic markers necessary for the production of adrenaline (Phillips *et al.* 2001). These PNMT-containing RVLM neurons are termed the C1 cell group (figure 2*a*). 'A' neurons (e.g. A1 neurons in the brainstem, A6 in the locus coeruleus or A10 that form the substantia nigra) lack PNMT and perform crucial functions throughout the neuraxis from the brainstem to the retina. 'B' neurons (B1, B2 and B3) synthesize serotonin and are located in the midline. Initially, it was believed that C1 neurons (Goodchild *et al.* 1984; Ross *et al.* 1984) were responsible for regulating sympathetic vasomotor pathways through the release of adrenaline in the spinal cord, but it eventually became clear that both C1 and non-C1 neurons also release glutamate. The actual role of adrenaline released at the level of sympathetic preganglionic neurons still remains unclear (Bolme *et al.* 1974). The possibility exists that it exerts complex effects depending on the post-synaptic

receptor present, and if an inhibitory interneuron is interposed (Shi *et al.* 1988; Coote & Lewis 1995).

3. PHYSIOLOGICAL REGULATION OF BLOOD PRESSURE AND RESPIRATION

The objectives in the regulation of blood pressure and the circulation of blood to specific organs at specific times are related to the objectives of ventilation. The prime objective of the cardiovascular system is to ensure an adequate flow of blood and plasma through the various organs so as to achieve goals that include: removal of carbon dioxide and delivery of oxygen (pulmonary), delivery of local and systemic hormones (renal, adrenal pituitary and many others), delivery of metabolic waste to the kidney and, as a consequence, of these activities the maintenance of a normal electrolyte and fluid-balance status. It is not possible in this short review to elaborate in detail the synchrony necessary for all of the bodily functions in the maintenance of homeostasis. Ventilation on the other hand is crucial for moment-to-moment acid–base control and oxygenation, as well as other functions such as vocalization.

In order to achieve the objectives remarked upon earlier, three components are coupled in order to govern normal activity: afferent, integrative and motor.

(a) *Afferent pathways to the autonomic nervous systems*

The peripheral and central systems that provide a motor output to blood pressure and breathing pathways sample information from sensory neurons that can be in the periphery (e.g. baroreceptors, figure 1*a*,*b*) or central nervous system (e.g. chemoreceptors) and then relay this information to the autonomic centres in the brainstem that generate tone and bursting activity. Baroreceptor afferent pathways (figure 1*a*,*b*) arise as nerve endings on the adventitia of the aortic arch or carotid sinus (Ciriello 1983; McDonald 1983; Pilowsky & Goodchild 2002); when blood pressure increases, baroreceptor nerves increase their firing rate. The information is then transmitted to the medial subnucleus of the nucleus of the solitary tract: a nucleus in the dorsomedial medulla that integrates (Smith *et al.* 2002) information from many sources and relays it to many places in the central nervous system including the ventral medulla. The fidelity of transmission in this pathway is excellent. It was recently reported that neurons receiving inputs from aortic arch baroreceptors have properties that are consistent with inputs arising from a single branched axon, a finding supported by anterograde tracing (Andresen & Peters 2008). These and other findings demonstrating the presence of ionotropic glutamate receptors (Aicher *et al.* 2002) suggest that fast neurotransmitters coupled to ligand-gated ion channels underlie these phenomena. The electrophysiological and morphological data are also supported by pharmacological data demonstrating that the baroreceptor reflex is blocked following microinjection of antagonists to glutamate ionotropic receptors into the medial nucleus tractus solitarius (NTS) (Gordon & Leone 1991). Other
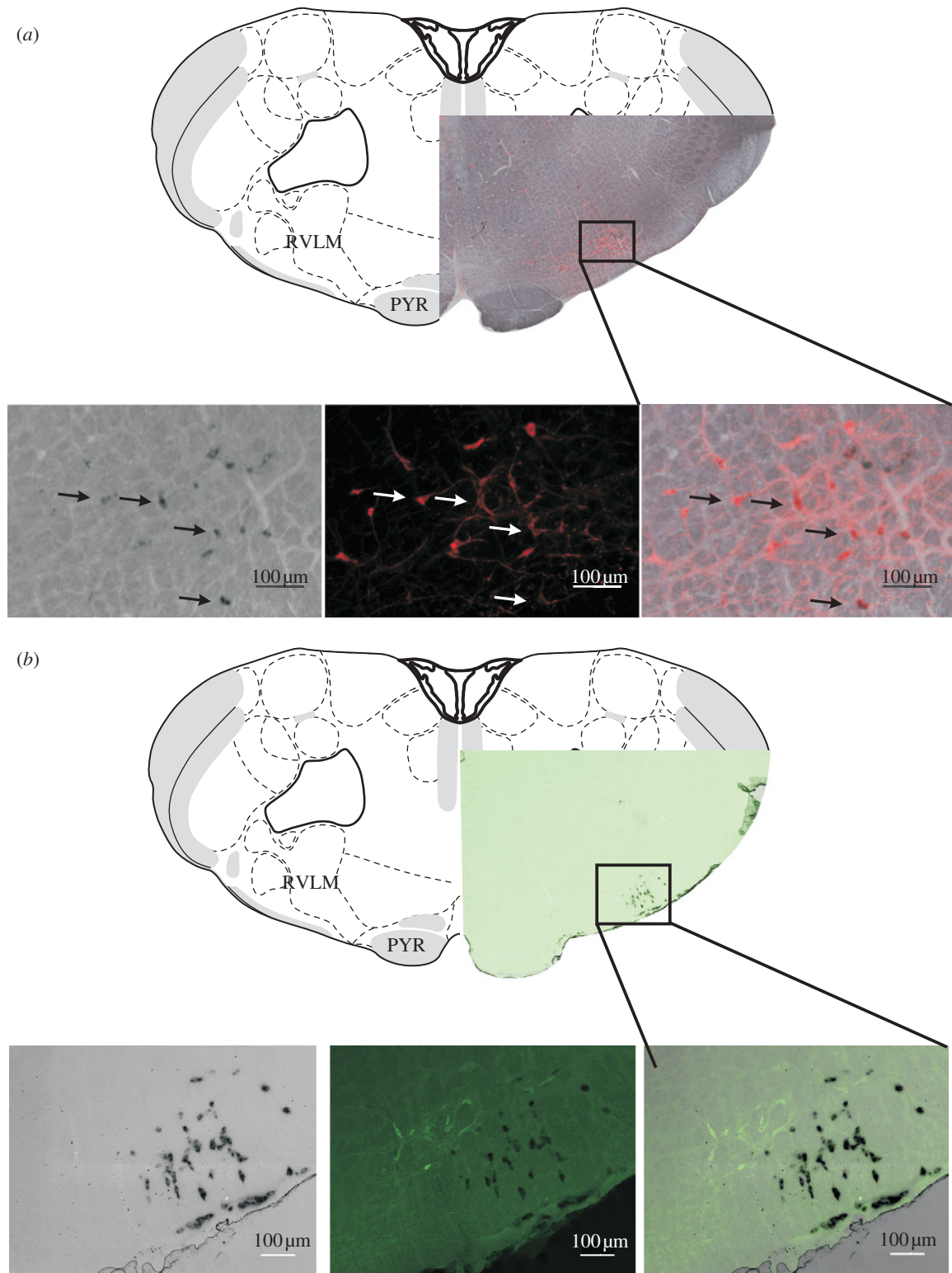
Figure 2. Neurotransmitter phenotypes in the RVLM (PYR, pyramidal tract). (*a*) Neurons in the RVLM that express PNMT (*in situ* hybridization—black) and are also immunofluorescent for tyrosine hydroxylase (red). The third panel shows a merged image and demonstrates colocalization in many of the cells. The drawing is taken from map 59 of Swanson (1998). Note that the pyramidal tract is present but the olivary nucleus is absent. Note also that both the rostral pole of the nucleus ambiguus and the caudal pole of the facial nucleus are present. These ventral landmarks define the rostrocaudal location of the RVLM (as indicated by the boxed area). (*b*) Galanin (pre-progalanin-expressing—black) neurons in the retrotrapezoid nucleus are close to and partly intermingled with tyrosine hydroxylase (green) immunofluorescent neurons in the RVLM.

evidence suggests that neuropeptides such as somatostatin may act as powerful longer-acting modulators of function at these sites (Chan *et al.* 1992). This means that while fast neurotransmission is essential for the full expression of the reflex, it is definitely not the case that the involvement of other regulators of cellular activity such as peptides is precluded. What remains to be determined is when and in what

situations all of these neurotransmitters are released. These questions are clearly not restricted to the neural regulation of the cardiovascular system.

(b) *Cardiorespiratory integration*

A great deal has been written on this topic (Baekey *et al.* 2008). Respiratory modulation of sympathetic nerve activity was first convincingly demonstrated in recordings of sympathetic nerve activity in 1932 (Adrian *et al.* 1932). The morphological substrates that might enable such a phenomenon to occur are a connection between neurons with respiratory modulation and those responsible for cardiovascular regulation. Such connections have been reported in cats (Pilowsky *et al.* 1994b) and rats (Pilowsky *et al.* 1992; Sun *et al.* 1997). Barosensitive neurons in both the rostral (Haselton & Guyenet 1989; Miyawaki *et al.* 1995) and caudal ventrolateral medulla (Mandel & Schreihofer 2006) are known to have a respiratory modulation that is most probably derived centrally because it is not locked to the phase of the ventilator in vagotomized and paralysed animals.

The main sites of integration of central cardiorespiratory regulation are located in the ventral medulla oblongata close to the facial nucleus and then caudally to the junction of the brainstem and spinal cord. The effects of chemical activation of brainstem cardiovascular sites are diverse; in the RVLM, glutamate is pressor and sympathoexcitatory while more caudally, depressor and sympathoinhibitory responses are obtained (Ross *et al.* 1983; Goodchild *et al.* 1984; Pilowsky *et al.* 1985; Guyenet *et al.* 1989). Chemical inhibition of these sites causes opposite effects and blocks the aortic nerve baroreceptor reflex (Willette *et al.* 1984; Pilowsky *et al.* 1985). Approximately 1 mm caudally lie the GABAergic neurons of the CVLM that are an integral part of the sympathetic baroreflex (Schreihofer & Guyenet 2003). In the most caudal parts of the brainstem—close to the cervical spinal cord—potent pressor and sympathoexcitatory effects can be elicited by chemical stimulation (Seyedabadi *et al.* 2006). The RVLM is considered to be absolutely crucial for tonic control of autonomic function and for the regulation of almost all autonomic reflexes (figure 1a–d). Although destruction of the RVLM completely eliminates sympathetic tone and reflexes, it is not the only site from which independent increases in sympathetic activity can be obtained; even after complete destruction of the RVLM, stimulation of the medullo-cervical pressor area can still evoke pressor and sympathoexcitatory effects (Seyedabadi *et al.* 2006).

There is still no consensus on how basal sympathetic tone is maintained. There are at least three possibilities. First, neurons in the RVLM may have membrane properties that cause them to fire at a particular rate at all times. Second, the activity of neurons in the RVLM may simply represent the sum of all inputs at any one time, and third, activity may be derived from the neurons in the RVLM as a combination of both possibilities. In fact, appealing though the first possibility is, the evidence for it as the mainstay of activity generation remains uncertain. In brainstem slices from neonatal rats, 50 per cent of C1 neurons have pacemaker properties. The

pacemaker properties are voltage dependent and not dependent on synaptic input. The pacemaker properties were attributed to the presence of a persistent sodium current (Kangrga & Loewy 1995). The second possibility also holds some appeal. In adult anaesthetized rats, sharp intracellular recording of bulbospinal neurons, inhibited by baroreceptor input, revealed that the neurons—many of which were C1 neurons—were continuously bombarded by inhibitory and excitatory post-synaptic potentials, but without any sign of pacemaker properties (Lipski *et al.* 1995, 1996). Calcium channels of all types also appear to be important in the normal functioning of C1 neurons and their ability to respond to a range of metabotropic neurotransmitters makes them attractive candidates in this regard (Li *et al.* 1998; Miyawaki *et al.* 2003). Perhaps the likeliest explanation of how these neurons operate is that they do have intrinsic biophysical properties that enable them to generate activity in certain circumstances, but that at most times they are regulated by external inputs to such an extent that these properties are less apparent. In certain preparations, such as slices from the neonatal rat, perhaps these other properties may become more evident. The recently described neurons in the retrotrapezoid nucleus (RTN; figures 1a,c and 2b) that are believed to be exquisitely chemosensitive have also been reported to have pacemaker-like activity in slices (Guyenet 2008). The pH sensitivity in both serotonin and RTN neurons is thought to be mediated by a K^+ channel (TASK—in the case of serotonin neurons since the pH sensitivity is abolished in TASK knockout animals Mulkey *et al.* 2007b).

Other medullary sites, including neurons in the midline, may also be important in the control of blood pressure (Minson *et al.* 1987), with activation of sites towards the midline medulla that contain serotonin neurons causing an increase in blood pressure that is associated with a release of serotonin in the spinal cord (Pilowsky *et al.* 1986a,b).

(c) *Respiratory integration*

In contrast to the uncertainty about how rhythmogenesis is generated in sympathetic outflow, there is greater consensus with respect to the respiratory system. As with the cardiovascular system, a column of nuclei present in the ventrolateral medulla is essential for the elaboration of normal (eupnoeic) respiratory activity. Put simply, phasic respiratory activity in different motor outputs is achieved through the sequential activation of populations of neurons that fire in the inspiratory or the expiratory phase. Both inspiratory-active and expiratory-active neurons may be either inhibitory or excitatory, depending on their neurotransmitter content and the precise phase of inspiration or expiration in which they are active. The key populations are the Bötzinger and pre-Bötzinger neurons (Smith *et al.* 1991; Sun *et al.* 1998; Koshiya & Smith 1999). Neurons in the preBötzinger region, particularly, have the electrical properties necessary to generate rhythm, and the morphological properties (extensive axon collateral arborizations) required to compose and distribute the generated activity into a

form appropriate to the relevant output pathways (Pilowsky *et al.* 1990*b*). Thus, the motoneurons of the larynx will cause vocal cord dilation just prior to the start of diaphragmatic contraction (Berkowitz *et al.* 1999*a*,*b*). The ventrolateral medulla is not the only brainstem site that may be important in respiratory regulation; recently a site in the midline between the caudal poles of the facial nucleus was reported where chemical excitation potently inhibits respiration (Verner *et al.* 2004, 2008) with little effect on blood pressure. The physiological significance of this site remains to be determined. Conceivably, this site is the endogenous source of the substance P that can influence respiratory neurons (Holtman *et al.* 1984; Gatti *et al.* 1999; Guyenet & Wang 2001; Sun *et al.* 2003; Mulkey *et al.* 2007*a*). Medullary nuclei outside the RVLM may also play a role in respiratory control. In particular, serotonergic neurons in the midline (Severson *et al.* 2003; Richerson 2004) and noradrenergic neurons (Li & Nattie 2006) may also play a role in responding to changes in chemoreceptor activation and transmitting this information to cardiorespiratory regulatory regions. Many authors have examined the importance of supra-medullary regions on respiratory regulation (Dawid Milner *et al.* 2003; Voituron *et al.* 2005); these regions will not be discussed here.

(d) *Motor pathways*
The final step following generation of activity is to distribute the information generated to the relevant motor output pathways.

(i) *Respiratory system*
Motor output pathways in the respiratory system are relatively uncomplicated in that there is a direct connection between a respiratory-generating neuron and a motoneuron, or there is an interposed pre-motoneuron that in turn activates a motoneuron. The axons of respiratory motoneurons may be relatively uncomplicated with either few (Hilaire *et al.* 1983; Lipski *et al.* 1985; Pilowsky *et al.* 1990*a*) or extensive recurrent collateral arborizations (Hilaire *et al.* 1983).

(ii) *Cardiovascular system*
The terminology 'pre-motoneuron' or pre-sympathetic neuron is also used in cardiovascular regulation, but the nature of the physiological role played by all of the pre-sympathetic neurons is uncertain. The neurons in the RVLM that are spinally projecting and barosensitive are often depicted in diagrams as simple neurons that project to the spinal cord where they excite sympathetic preganglionic neurons. Commonly, when discussing the tonic and reflex regulation of the sympathetic nervous system, the main focus is the RVLM for reasons noted above; however, there are at least five other areas (above the spinal cord) that project caudally and are thought to be pre-sympathetic (Jansen *et al.* 1992; Krout *et al.* 2003; Seyedabadi *et al.* 2006). As noted above, this is part of the story, but by no means all of it. One very careful anterograde tracing study that used viral tracing combined with specific promoters so as to target only C1

neurons reported that while the expected dense projection to the intermediolateral cell column was indeed present, other targets also received an innervation, including a sparse but definite projection to the contralateral RVLM (Card *et al.* 2006). Evidence for an intramedullary projection of C1 neurons was also provided by Madden *et al.* (1999). who reported that following selective unilateral lesion of C1 neurons with antibodies to the adrenergic membrane protein dopamine-β-hydroxylase conjugated to the ribosomal neurotoxin saporin, there was a loss of some C1 neurons on the contralateral side. One plausible explanation for such data is that there is network activity or at least coordination between the two pre-motor cell groups in the same way as occurs in the respiratory system. However, there are other explanations—including the possibility that C1 neurons are indeed auto-active in some circumstances (Kangrga & Loewy 1995; Li *et al.* 1995), or that RVLM neurons simply act to integrate central and peripheral inputs without requiring intrinsic mechanisms to maintain activity. Clearly, much more work is needed to understand the extent to which these different possibilities are important parts of the whole (Lipski *et al.* 2002). At the moment, what is lacking is convincing evidence of a phenomenon of centrally generated sympathetic rhythms that require explanation in the same way that respiratory rhythms are needed. In fact, removal of baroreceptor inputs in conscious rats eliminates peaks in frequency spectra in arterial pressure apart from that caused by respiration (Kunitake & Kannan 2000), suggesting that there is no intrinsic oscillator that affects sympathetic output.

Evidence does exist for connections between functionally characterized sympathetic pre-motor neurons and sympathetic preganglionic neurons, although the precise role of individual connections between pre-motoneurons at supraspinal levels and sympathetic preganglionic neurons in the spinal cord is still uncertain. Monosynaptic connections using correlation techniques have been reported in cats (McAllen *et al.* 1994) and rats (Oshima *et al.* 2006). Recently, this result has been confirmed by spike-triggered averaging experiments combining extracellular recording from brainstem neurons with whole-cell patch clamp recording from sympathetic preganglionic neurons (Oshima *et al.* 2008). Of the many types of preganglionic neuron (Jänig & McLachlan 1992), cardiovascular sympathetic preganglionic neurons (as defined by the presence of bursts of excitatory post-synaptic potentials (EPSPs) in phase with phrenic nerve discharge and a slowly conducting axon) only form a small proportion of the total number of sympathetic preganglionic neurons in the spinal cord (approx. 7%). These cardiovascular sympathetic preganglionic neurons generally have small somata, but extremely extensive dendritic trees (Pilowsky *et al.* 1994*a*). The large amount of axonal arborizations from PNMT immunoreactive terminals in the intermediolateral cell column combined with the extensive dendritic arborizations of sympathetic preganglionic neurons suggests the possibility of considerable divergence in the bulbospinal input pathways. However, the electron-microscopic evidence in favour of an extensive input to sympathetic

preganglionic neurons is not strong (Milner *et al.* 1988*a*,*b*; Llewellyn-Smith *et al.* 1991). There are strong teleological arguments in favour of this idea (ensuring that general vasoconstriction occurs where needed throughout vascular beds), but equally strong counterarguments (in that organ-specific vasoconstriction is also needed and that sympathetic activity can be controlled differentially). Clearly, additional experimentation—and possibly novel tools—is needed.

4. NEUROTRANSMISSION

It is generally accepted that within the central nervous system three ionotropic neurotransmitters are primarily responsible for regulating activity in cardiorespiratory pathways *viz.* glutamate, GABA and glycine. Others, such as acetylcholine (Shao & Feldman 2001) and serotonin may also act on ligand-gated ion channels to exert rapid changes in membrane potential, but the effect of these latter neurotransmitters—as well as that of others—discussed below, is principally exerted on metabotropic receptors that are coupled to G proteins (Martin 1992; Pelat *et al.* 1999; Padley *et al.* 2005). Cannabinoids (Padley *et al.* 2003), gases such as nitric oxide (Zanzinger *et al.* 1995; Kishi *et al.* 2002; Gao *et al.* 2008) and other novel mediators are also part of the environment that influences the long- and short-term activity of cardiorespiratory neurons.

The 'simplistic' understanding of how G-protein-coupled receptors (GPCRs) work is clouded by the discovery that dimerization (both between the same receptor type and between different receptor types) can lead to activation of entirely different signal transduction pathways with effects that are the reverse of those normally seen (e.g. Duran-Prado *et al.* 2008). This is not the place for a detailed discussion of the complexities of G-protein signalling (Achour *et al.* 2008), but it is important to note that since they are the largest family of receptor-coupled proteins, abductive reasoning (Haig 2008) suggests that they play a very significant role in modulating the physiological interactions of neurons that are crucial for cardiorespiratory regulation.

In fact, almost all neurotransmitters exert their effects through multiple receptors that may be either ionotropic, metabotropic, inhibitory or excitatory. This ability of a neurotransmitter to exert more than one effect depending on the receptor expression profile of its target can be termed 'pleiotropy'. The pleiotropic effects of neurotransmitters do not necessarily disprove Dale's principle as elaborated by Eccles (Burke 2006), that a neuron will release all of its neurotransmitters at all of its synapses, but it does seem to diminish the utility of the hypothesis if it means that the response to the release of such neurotransmitters may be completely different depending on the pre- or post-synaptic receptor profile. The hypothesis is further diminished by some more unusual circumstances where spatial and temporal release segregation of neurotransmitters from a neuron occurs (Sossin *et al.* 1990).

Because so much of the moment-to-moment control of neural networks appears to be under the control of ionotropic receptors that are operated by glutamate, GABA and glycine, we have attempted to define the role played by other neurotransmitters with a combination of microinjection into specific brain nuclei and analysis of specific reflexes. To achieve this, we generally use an adult 'semi-reduced' *in vivo* preparation in which only the sympathetic nervous system is active (achieved by vagotomy and atropine administration). We then record from at least one sympathetic nerve (generally the greater splanchnic) and the phrenic nerve, and activate baroreceptors (figure 1*a*,*b*), chemoreceptors (figure 1*c*) or somatic afferent neurons (figure 1*d*) before and after administration of agonist and antagonist agents. In this preparation, changes in heart rate may also represent a surrogate sympathetic output to the heart (integrating both direct neural input and influences from circulating catecholamines) as the vagi are cut.

Our findings reveal that there is a clear discrimination of different receptors on different types of reflexes, supporting our initial hypothesis. Here I will briefly survey some of these findings in relation to some of the neurotransmitters that we have examined.

5. SEROTONIN IN CARDIORESPIRATORY REGULATION

Serotonin is a compound that has entered popular consciousness because of its positive effects on mood in patients suffering from depression. It is present in a restricted population of neurons in the brainstem, but the influence of these neurons is felt throughout the central nervous system. In the spinal cord, serotonin densely innervates phrenic motoneurons (Holtman 1988; Holtman *et al.* 1990; Pilowsky *et al.* 1990*a*) and sympathetic preganglionic motoneurons (Pilowsky *et al.* 1995*a*). It is released in the spinal cord following activation of cell bodies in the brainstem (Pilowsky *et al.* 1986*a*,*b*) and plays a role in plasticity in long-term potentiation of phrenic neural activity following intermittent hypoxia (Baker-Herman & Mitchell 2002). The precise mechanism of action of serotonin is not established in all systems because of its many receptors (Hoyer *et al.* 1994) and because of the many neurotransmitters that are coreleased with it (Jansen *et al.* 1995).

Does the anatomical finding of serotonin in the different spinal nuclei suggest specific functions? The answer here is unfortunately no. The neurons that provide the serotonergic input must come from the caudal Raphé as this is the only source of such cells (Pilowsky *et al.* 1995*b*; Lalley *et al.* 1997; Mason 1997; Richerson *et al.* 2001; Ootsuka *et al.* 2004). The many studies conducted on Raphé neurons suggest that individual cells may influence functions as diverse as control of pain, blood pressure and motor function. Furthermore, it seems that most serotonergic neurons contain other neurotransmitters including peptides and amino acids (Jansen *et al.* 1995) so that it is possible—even quite likely—that the majority of the effects mediated by Raphé neurons are not due to the release of serotonin. Thus, working out which Raphé neurons release which neurotransmitters, and under what circumstances, to mediate which effects, are all mysteries. The advantage of furthering our knowledge in this

regard is that it may lead to the development of therapies that have greater specificity in targeting functions. One possibility is that serotonergic neurons play a system-wide role in raising tone in autonomic regions so that individual reflexes or functions become more or less sensitive depending on the activity of the inputs as suggested by workers using Fos studies and carbon dioxide exposure, who found a widespread activation of serotonin neurons (Haxhiu *et al.* 2001). Recently, it was reported that serotonin directly excited chemosensitive neurons, but that this occurred via a mechanism that was distinct from the ability of these chemosensors to detect change in pH (Mulkey *et al.* 2007*a*). The idea of a widespread role in modulating autonomic functions is further supported by reports that serotonergic, and noradrenergic, inputs are excitatory to hypoglossal motoneurons, and that the withdrawal of these inputs may be an underlying factor in muscle atonia in rapid-eye-movement sleep (REM sleep; Fenik *et al.* 2005). Moreover, in mice that lack the serotonin transporter, there is a disturbance in REM sleep compared with control mice (Wisor *et al.* 2003). A differential serotonergic input onto laryngeal motoneurons also exists, with constrictor motoneurons receiving a greater input than dilator motoneurons (Sun *et al.* 2002; Berkowitz *et al.* 2005).

In rats, if the serotonin 1a (5HT1a) receptor agonist 8-hydroxy-di-*n*-propylamino tetralin is microinjected bilaterally into the RVLM, there is a fall in arterial blood pressure and sympathetic blood pressure along with a decrease in the amplitude of phrenic nerve discharge (Miyawaki *et al.* 2001). The effect is by no means as large as the potent effects that can be achieved with GABA or glutamate, and on average, blood pressure only fell by 13 mm Hg. Despite this apparently modest effect on resting parameters, the effects on reflex function were profound. The two peak somatosympathetic reflexes seen in ensemble averages of splanchnic nerve activity were completely abolished, while baroreceptor function and hypoxia (10 s of 100% nitrogen instead of 100% oxygen) were unimpaired. All effects were prevented by pre-treatment with the 5HT1a antagonist NAN-190, which by itself had no effect on any measured parameters (Miyawaki *et al.* 2001).

6. CATECHOLAMINES IN CARDIORESPIRATORY REGULATION

Catecholamines are also major players in the central regulation of cardiorespiratory neurons. Mainstays of the chemotherapy of hypertension such as alpha-methyldopamine, clonidine and moxonidine are thought to act through neurons in the RVLM. Clonidine, via activation of central alpha-2 receptors, causes a decrease in ventilation and is hypotensive and sympatholytic (Bolme *et al.* 1974; Koshiya & Guyenet 1995; Guyenet 1997; Grubb *et al.* 1998). Interestingly, with respect to cardiorespiratory integration, the post-inspiratory phase of sympathetic nerve activity is more sensitive to the sympatholytic effects of clonidine than in the inspiratory phase (Koshiya & Guyenet 1995), suggesting that sympathetic activity and arterial pressure are preserved

preferentially during the inspiratory period; an effect that may serve to enhance oxygen delivery to tissue.

7. PEPTIDES IN CARDIORESPIRATORY REGULATION

Why look at peptides and other colocalized neurotransmitters if amino acids do all the work? The simple answer is that all of the 'work' is not done by short-acting transmitters. More importantly, there appears to be a segregation of function according to the different metabotropic transmitter receptors that are activated or inhibited. This means that one neuropeptide may selectively antagonize the somatosympathetic reflex (figure 1*d*) but not the baro- (figure 1*b*) or chemo-reflex (figure 1*c*). This is the general theme that we have been pursuing in our laboratory over the past 10 years.

It is not possible to survey all the peptides that have been implicated in cardiorespiratory regulation, so I will aim to mention only those for which there is at least some physiological or pharmacological evidence for a role in cardiorespiratory regulation.

(a) *Opioids*

Opioids are one of the first classes of peptides discovered and have a long history, scientifically (two Nobel prizes), socially (drug addiction) and in the literature (Dorothy falling asleep in a field of poppies in the Wizard of Oz). In combining all three, we need look no further than the occasionally opium- (as well as cocaine-) addicted, forensic scientist of literary fame: Sherlock Holmes. Opioids are famous as centrally acting cardiorespiratory depressants.

As a class of ligands, opioids bind to receptors (GPCRs) on the cell membrane that are coupled to G proteins (usually Gi/o in the case of opioid receptors; Wettschureck & Offermanns 2005) both inside and outside the nervous system (Wu & Wong 2005). The effect of the activation of opioid receptors is almost uniformly inhibitory and the intracellular mechanisms that mediate the cellular hyperpolarization that causes this inhibition depends on the opening of potassium channels, and inhibition of adenylate cyclases, among other things.

C1 neurons are themselves opioidergic and receive opioid inputs (Stasinopoulos *et al.* 2000; Stornetta *et al.* 2001). To address the relationships between opioid systems and cardiorespiratory neurons, we and others have used a range of approaches, including histological, pharmacological, electrophysiological and physiological. These studies reveal many facets of the way in which opioids can interact with cardiorespiratory neurons. Many inputs to C1 pre-sympathetic neurons are immunoreactive for the delta-opioid receptor, although the pre-sympathetic neurons themselves are not (Stasinopoulos *et al.* 2000). Microinjection of the delta agonist [D-Pen[2;5]]-enkephalin (DPDPE) has complex effects on central cardiovascular regulation (Miyawaki *et al.* 2002). Immediately following microinjection into the RVLM bilaterally, DPDPE causes a fall in arterial blood pressure and a reduction in lumbar sympathetic nerve activity (LSNA). The reduction in LSNA is principally

associated with an almost complete loss of the post-inspiratory peak normally seen in the activity of this nerve. Testing of reflexes reveals that opioids that exert their effects via delta receptors have quite different effects from those seen following mu-agonist administration. While the somatosympathetic reflex is abolished, the sympathetic baroreflex and the chemoreflex (ventilation with 100% nitrogen for 10 s) are completely unaffected. A similar selective reduction in the somatosympathetic reflex can be achieved with hypercarbia (Makeham *et al.* 2004).

Mu-opioid receptors in the RVLM exert quite different effects when activated: arterial blood pressure and sympathetic nerve activity also fall, and the chemoreceptor reflex is also unaffected. However, in contrast to delta receptor agonism in the RVLM, mu-opioid agonism causes an attenuation of the sympathetic baroreceptor reflex with no effect on the somatosympathetic reflex (Miyawaki *et al.* 2002). Mu-opioid receptors are found both pre- and post-synaptically on neurons in the RVLM with a very marked post-synaptic preponderance (Aicher *et al.* 2001). Such a morphological arrangement would permit mu agonists to occlude baroreceptor inputs arising from inhibitory neurons in the caudal ventrolateral medulla (figure 1*b*); it remains to be determined in which of the afferent pathways mu receptors are found, but if they are absent from those mediating the somatosympathetic reflex and the chemoreceptor reflex, then the relative lack of effect on these reflexes would be more easily understood.

(b) *Neuropeptide Y*
Neuropeptide Y (NPY) is expressed in the RVLM (Agnati *et al.* 1988) and is colocalized with many C1 neurons that project to the hypothalamus (Li & Ritter 2004), but in only approximately 9 per cent of those that project to the spinal cord (Blessing *et al.* 1987; Stornetta *et al.* 1999). Most studies on a potential physiological role for NPY in the spinal cord have focused on its importance in mediating painful stimuli (Shi *et al.* 2006). Recently, it was found that intrathecal NPY will attenuate somatosympathetic (Kashihara *et al.* 2008) and noxious stimuli (Mahinda & Taylor 2004). Some authors report that NPY will induce pressor responses when delivered intrathecally, although this response is not always found (Mahinda & Taylor 2004). In any event, it is not known if any physiologically relevant pressor response to spinal NPY release is mediated by such a small population of bulbospinal sympathoexcitatory neurons, or by activation of ascending nociceptive pathways.

(c) *Apelin*
Apelin is a relatively new peptide. It acts through its own GPCR known as the APJ receptor and has effects peripherally and centrally. Despite some sequence similarities with the angiotensin II type 1 receptor, the two peptides do not bind to the others receptors. Generally, the effects of apelin in the periphery (Chandrasekaran *et al.* 2008) are opposite to those of angiotensin II, with apelin causing effects that are hypotensive. Few studies have examined possible

roles for apelin in the central regulation of the cardiorespiratory system. Microinjection studies from our laboratory suggest that apelin has effects on blood pressure and phrenic nerve discharge in two key cardiorespiratory nuclei in the brainstem, *viz.*, the NTS and the RVLM (Seyedabadi *et al.* 2002). A physiological role for apelin remains to be determined.

(d) *Angiotensin II*
Angiotensin peptides are among the most extensively investigated peptides in central cardiorespiratory regulation. Most studies focus on the effect of angiotensin II on angiotensin type-1 receptors. The roles of angiotensin II in homeostasis are broad. Here I will focus on only a few. Following intravenous injection, one of the effects of angiotensin II is to activate neurons in the circumventricular organs. This binding activates neuronal pathways that project from the hypothalamus either directly to the sympathetic motor pathways in the spinal cord or via a synapse in the RVLM (Li *et al.* 1992). The phenotype of the input to RVLM may be in part cholinergic (Kubo *et al.* 2002), but is not completely clarified. Qualitatively, the effect of vasoconstriction with angiotensin II is quite different from that seen with a peripherally acting alpha-1 agonist such as phenylephrine. Despite very high levels of arterial blood pressure that can be achieved with angiotensin II, sympathetic nerve activity is not abolished and baroreceptors are still effective in lowering arterial blood pressure and suppressing sympathetic nerve activity (McMullan *et al.* 2007). Equally surprising is the finding that some bulbospinal baroinhibited neurons in the RVLM are not inhibited following intravenous angiotensin II and some baroinhibited neurons are actually activated (McMullan *et al.* 2007).

Angiotensin II receptor activation is also known to exert clear effects in different parts of the ventrolateral medulla. Microinjection into the caudal ventrolateral medulla causes vasopressin release, although effects on respiration were not documented in that study (Allen *et al.* 1990). Microinjection of angiotensin II into the RVLM increases arterial blood pressure, an effect that is mediated by MAP kinase in normotensive rats and by both MAP kinase and PI3 kinase in hypertensive rats (Seyedabadi *et al.* 2001). When applied to individual C1 neurons in RVLM, angiotensin II causes a depolarization mediated by closure of potassium conductance (Li & Guyenet 1995, 1996). In a bath preparation, application of angiotensin II to the brainstem excites neurons, which in turn project to, and excite, sympathetic preganglionic neurons (Oshima *et al.* 2008).

(e) *Other peptides*
As noted earlier, many other peptides play an important role in the tonic and reflex regulation of the cardiorespiratory systems, including substance P (Gilbey *et al.* 1983; Makeham *et al.* 2001, 2005), pituitary adenylate cyclase-activating polypeptide (PACAP; Farnham *et al.* 2008), somatostatin (Burke *et al.* 2008), galanin (Stornetta *et al.* 2009; figure 2*b*), which is present in many chemosensitive neurons in the retrotrapezoid nucleus, and thyrotropin-releasing hormone (Murphy *et al.*

1995; Sun *et al.* 1995, 1996). The effects of these neuromodulators are site specific and in most cases their physiology is still very poorly understood.

8. ANSWERS TO QUESTIONS

Can we provide any insights into the questions posed at the beginning? In answer to the question of why cardiorespiratory neurons appear to have multiple neurotransmitters, it seems that we are not well advanced. Can we tell if different brainstem neurons that contain different populations of neurotransmitters are targeted to different functional populations of motor outputs? The evidence in support of this proposition is weak, although we do know, for example, that C1 neurons are not respiratory neurons (Pilowsky *et al.* 1990b). Within the separate populations of cardiovascular and respiratory neurons, finer discrimination still eludes us. Can we associate different neurochemicals to specific populations of neurons? Here we are doing a little better, some C1 neurons (at least 26%) are definitely bulbospinal and inhibited by baroreceptors (Lipski *et al.* 1995). It also seems likely that most, if not all, bulbospinal C1 neurons are PACAP containing (Farnham *et al.* 2008) and that approximately 18 per cent of C1 neurons contain substance P (Li *et al.* 2005). Substance P (Solomon *et al.* 1999) and PACAP (Farnham *et al.* 2008) are both known to be sympathoexcitatory when injected intrathecally. The extent to which different populations of neurochemically identified neurons define specific functional pathways and the physiological roles that they may play remain mysterious and a challenge for future studies.

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