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Identification of neurotransmitters and co-localization of transmitters in brainstem respiratory neurons

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Abstract

Identifying the major ionotropic neurotransmitter in a respiratory neuron is of critical importance in determining how the neuron fits into the respiratory system, whether in producing or modifying respiratory drive and rhythm. There are now several groups of respiratory neurons whose major neurotransmitters have been identified and in some of these cases, more than one transmitter have been identified in particular neurons. This review will describe the physiologically identified neurons in major respiratory areas that have been phenotyped for major ionotropic transmitters as well as those where more than one transmitter has been identified. Although the purpose of the additional transmitter has not been elucidated for any of the respiratory neurons, some examples from other systems will be discussed.

Keywords

neurotransmitter co-localization; in situ hybridization; peptides; respiration; juxtacellular labeling

1. Introduction

Identification of the major ionotropic, fast synaptic transmitter used by a neuron is a major step forward in understanding the role of the neuron in a functional network. The neurons in the respiratory network were initially characterized as excitatory or inhibitory by using electrophysiological techniques allowing identification of fast inhibitory or excitatory post-synaptic potentials elicited from the identified neuron. In addition to the excitatory or inhibitory nature of the major transmitter, the morphology of some of these physiologically identified neurons was examined, yielding more information about how the neuron might participate in the respiratory network by studying its dendritic fields and axonal projections. A more recent method for identifying the major transmitter is through the use of in situ hybridization (ISH) for definitive markers of glutamatergic, glycinergic or GABAergic transmission combined with cellular labeling of a physiologically identified respiratory neuron. The technique of ISH can be combined with immunocytochemistry and/or double ISH to determine other phenotypic markers for respiratory neurons. ISH has been used to find that other neurotransmitters or neuropeptides with slower kinetics are sometimes co-localized with fast transmitters. In this review, the respiratory areas of the brainstem will be surveyed in terms of definitive

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characterization of a fast ionotropic transmitter and also those that have been identified with co-localized multiple transmitters.

Most neurons in the brain use glutamate, GABA, and/or glycine as their major ionotropic neurotransmitter. For instance, virtually all spontaneous miniature post synaptic potentials in brainstem slices are blocked by a cocktail of glutamatergic, GABAergic and glycinergic antagonists (Hayar and Guyenet, 1998, 1999; Lin et al., 1998; Doyle and Andresen, 2001). Electron microscopy also reveals that nearly all terminals in brainstem regions contain one of these three transmitters (Llewellyn-Smith et al., 1995, 2001). Although antibodies have been available for GABA, glycine and glutamic acid decarboxylase (GAD), the enzyme present in all GABAergic neurons, these markers are found most prominently in neuronal processes and not in cell bodies. Phosphate activated glutaminase (PAG) has been promoted as useful in identifying glutamatergic neurons but this enzyme has been found in Bötzinger neurons (glycinergic and not glutamatergic)(Pilowsky et al., 1997). Antibodies against glutamate itself have also been used to identify glutamatergic neurons. However, many neurons contain glutamate without it being used as a neurotransmitter, e.g. it is expressed in GABAergic neurons as a precursor of GABA and is used in many metabolic pathways (McKenna, 2007).

These drawbacks for identifying the major ionotropic transmitter can now be overcome by using labeled cRNAs to identify mRNAs coding for GAD 65/67 (Erlander et al., 1991; Esclapez et al., 1993), glycine transporter-2 (GlyT2) (Jursky et al., 1994; Jursky and Nelson, 1995) and three vesicular glutamate transporters (VGluts) (Bellocchio et al., 1998, 2000; Fremeau et al., 2001, 2002, 2004; Herzog et al., 2001, 2004; Varoqui et al., 2002; Oliveira et al., 2003). The major player in terms of markers for glutamatergic transmission in respiratory-related neurons is VGlut2, since in brainstem, VGlut1 is located predominantly in neurons supplying mossy fibers to the cerebellum (Hisano et al., 2002; Stornetta and Guyenet, 2005) and VGlut3 is limited to neurons in raphe magnus, parapyramidal raphe and dorsal and median raphe nuclei (Herzog et al., 2004; Nakamura et al., 2004; Stornetta et al., 2005) and in nitroxidergic neurons in the nucleus of the solitary tract (NTS) (Lin and Talman, 2005). The ISH method for using non-radioactive cRNA probes for GAD 65/67, GlyT2 or VGlut2 is performed using free-floating sections from paraformaldehyde fixed brains. Both digoxigenin and fluoroiso-thiocyanate (FITC) can be coupled to nucleotides and incorporated into cRNA probes. The resulting label from specific hybrids formed in the tissue is amplified by standard immunocytochemical methods. These probes can label neuronal somata with high signal-to-noise ratios and the labeling can be combined with other immunocytochemical and tract-tracing methods (c.f. (Stornetta et al., 2001, 2002a, 2004; Stornetta and Guyenet, 2005)).

Knowing which major ionotropic transmitter the cell uses, glutamate, GABA and/or glycine defines whether the cell is predominantly excitatory or inhibitory. By having information on other signaling molecules, e.g. neuropeptides, in addition to the major transmitter, one begins to gather a better “fingerprint” of a specific cell and perhaps an improvement in assigning the cell to a functional group. However, one must use some caution in trying to determine the function of a neuron based solely on a specific combination of phenotypic markers. One example of the limitations of determining function of brainstem neurons based on co-localization of phenotypic markers is the barosensitive bulbospinal cardiovascular premotor neurons of the ventrolateral medulla. Both VGlut2 and enkephalin have been identified in both C1 (tyrosine hydroxylase-TH-containing) and non-C1 cardiovascular premotor neurons (Stornetta et al., 2001, 2002b). There was no obvious difference between the C1 enkephalin neurons and non-C1 enkephalin neurons in their firing patterns, the response to blood pressure changes or in or in their spinal projections. However, the enkephalin neurons did tend to have a faster conduction velocity regardless of their co-localization with TH. The effects of opiates, glutamate and epinephrine on the sympathetic preganglionic neurons (SPNs) are quite different (although epinephrine as a C1 transmitter is controversial (Sved, 1989)) and these effects may

be mediated both pre- and post-synaptically (Backman and Henry, 1983; McCall, 1988; Dun et al., 1992, 1993; Krupp and Feltz, 1995; Miyazaki et al., 1998). Certainly the co-release of enkephalin and glutamate on SPNs could have complex effects; however, the functional significance of this finding in terms of cardiovascular regulation has not been elucidated. Also note that although the effects of neuropeptides such as enkephalin on cardiorespiratory functions have been extensively studied (Hassen et al., 1982, 1984; Feuerstein et al., 1983; Pfeiffer et al., 1983; Iasnetsov et al., 1984; Suzue, 1984; Harfstrand et al., 1985; Hurlë et al., 1985; Sessle and Henry, 1985; Van Bockstaele and Aston-Jones, 1995; Gray et al., 1999; Mellen et al., 2003; Onimaru et al., 2006; Barnes et al., 2007; Bodnar, 2007), their function when co-localized with major transmitters in brainstem neurons has not yet been determined (although see below for discussion of the co-release of these substances in other part of the CNS).

The first indications of the major ionotropic transmitter in respiratory neurons were found using spike-triggered averaging of membrane potentials recorded with intracellular electrodes in neurons identified by their firing pattern in relation to a respiratory output. If the membrane potentials were hyperpolarizing and chloride mediated, these would indicate either glycine or GABA transmission. If the membrane potentials were fast rising, short lasting depolarizations, these were most likely due to ionotropic excitatory (possibly glutamatergic) transmission. A good review of the knowledge obtained by these methods is summarized in Ezure (1990). A newer method for determining the major ionotropic transmitter uses the juxtacellular labeling technique (Pinault, 1996) to allow the phenotypic identification of physiologically characterized respiratory neurons. This method allows one to use extracellular recording which is much easier in whole animals than the intracellular recording technique. Juxtacellular labeling can be done in the whole animal in vivo, thus allowing the study of the intact system. Following the experiment, the animal can be perfused transcardially for optimal histology, surmounting many issues with fixation of slices or other reduced physiological preparations. This technique can also be used in reduced preparations. Using the juxtacellular technique, a neuron can be identified based on both projection (via antidromic activation from the area of projection) as well as its firing pattern in relation to a respiratory outflow (e.g. phrenic nerve activity). The Neurobiotin or biocytin deposited via juxtacellular labeling can be easily combined with other markers visualized with histochemical techniques such as in situ hybridization and immunohistochemistry (for further information on juxtacellular labeling see the review by Guyenet et al. (2004)).

The respiratory network includes the nucleus of the solitary tract (NTS) and a series of contiguous regions located in the ventrolateral medulla and extending into the dorsal pons. The respiratory network in the ventrolateral medulla is referred to as the ventral respiratory column (VRC) in groups designated from caudal to rostral as the caudal ventral respiratory group (cVRG), the rostral ventral respiratory group (rVRG), the preBötzinger complex (preBötC), the Bötzinger complex (BötC), the parafacial respiratory group (pFRG) and the retrotrapezoid nucleus (RTN) (see Fig. 1, for reviews and more thorough anatomical explanations see (Feldman, 1986;Ezure, 1990;Alheid et al., 2002)). Some respiratory neurons (mostly inspiratory) are located in the area of the NTS and are referred to as the dorsal respiratory group (DRG)(de Castro et al., 1994). A prominent group of respiratory-related neurons is located in the pons around the parabrachial nucleus (PBr) and including the Kölliker Fuse (KF) nucleus is called the pontine respiratory group (PRG) (Alheid et al., 2004;Duffin, 2004;Ezure and Tanaka, 2006). The expression (and co-expression) of neurotransmitters will be considered in these general areas.

2. Brainstem respiratory areas with identified major ionotropic transmitters

2.1 cVRG

This area beginning at the level of the obex (rostral to the calamus scriptorius) just caudal to the nucleus ambiguus and extending to the spinal medullary border, contains several different types of neurons with respiratory-related activity. Ezure and coworkers examined neurons with a firing pattern that decreased during expiration (termed expiratory-decrementing or E-DEC neurons) throughout the VRG and found vagal motor neurons in cVRG with E-DEC activity that were cholinergic (identified with immunostaining) and that did not contain either GlyT2 or GAD-67 mRNA (Ezure et al., 2003). The E-DEC cranial motor neurons are also not GABAergic (as determined by immunohistochemical detection of GAD) (Yamazaki et al., 2000; Okazaki et al., 2001). Another group of E-DEC neurons, some of which are located in the cVRG, have propriobulbar (a few additionally with bulbo-spinal) connections and are glycinergic as determined by juxtacellular labeling in combination with ISH for GlyT2 (Ezure et al., 2003). These neurons were not choline acetyltransferase (ChAT) immunoreactive, thus most likely not motor neurons. A few that were tested lacked GAD67 mRNA. Yamazaki and coworkers described a few GAD-immunoreactive neurons with different respiratory-related discharges in the cVRG including firing just following inspiration (post-I), firing increasing during inspiration (inspiratory-augmenting, I-AUG) as well as E-DEC; most of these neurons also contained NMDA receptors (detected by immunoreactivity) and were propriobulbar with no spinal or vagal projection (Yamazaki et al., 2000). Whether or not the E-DEC neurons found by Yamazaki's group are the same subset of inhibitory neurons described by Ezure and coworkers is uncertain due to the small sample sizes of both studies as Ezure admits he cannot exclude the possibility that some of the glycinergic E-DEC cells in the cVRG might also be GABAergic. Okazaki et al. (2001) found propriobulbar I-AUG neurons in cVRG that were GAD-immunoreactive, consistent with Yamazaki et al. Okazaki and coworkers (2001) also found GAD-immunoreactive, I-AUG neurons extending as far as 600 microns rostral to the obex (in rat) which would correspond to the rVRG.

Most of the neurons found in the cVRG with a firing pattern that increases during expiration (expiratory-augmenting, E-AUG) are excitatory (presumed glutamatergic although the exact phenotype has not been identified) pre-motor neurons projecting to the ventral horn neurons controlling abdominal and other expiratory muscles (Ballantyne and Richter, 1986; Arita et al., 1987; Iscoe, 1998).

2.2 rVRG

The rVRG, a collection of respiratory neurons located in the ventrolateral medulla, extends from the level of the obex to the rostral portion of the nucleus ambiguus (Ellenberger and Feldman, 1990). The pre-motor, I-AUG neurons in the rVRG have been characterized as excitatory by several different methods. Ono et al. (2006) used spike-triggered averaging to find EPSPs in laryngeal motoneurons elicited from I-AUG neurons in rVRG. Liu et al. (1990) found pharmacological evidence that inspiratory-related bulbospinal input to phrenic motor neurons was from excitatory amino acids. Stornetta et al. (2003b) found further evidence that bulbo-spinal I-AUG neurons are excitatory (glutamatergic). Neurons in the rVRG that project to the phrenic motor neurons in the ventral horn of the cervical spinal cord were identified by the retrograde transport of FluoroGold (FG) injected into the cervical spinal cord at C4-6 (Fig. 2A). These neurons were also labeled with riboprobes for VGlut2 and pre-pro-enkephalin (ENK). Sixty percent of the FG-labeled neurons were found to co-express both VGlut2 and ENK mRNAs (Fig. 2B-D). Biotinylated dextran amine (BDA) iontophoresed into the area of the rVRG (identified by recording of inspiratory-augmenting activity in the area) was transported to terminals found in cervical spinal cord ventral horn to co-express VGlut2 and enkephalin proteins by immunocytochemistry (Fig. 2E, F). Finally, 16/18 physiologically

identified I-AUG neurons contained VGlut2 mRNA and 14/14 I-AUG neurons contained ENK mRNA (Fig. 2G-J). These data support the idea that this population of bulbospinal I-AUG neurons of the rVRG are both glutamatergic and enkephalinergic.

The region of the ventrolateral medulla caudal to the preBötC extending to the forking rostral tips of the lateral reticular nucleus (LRN) is also known as the caudal ventrolateral medulla (CVLM) in the area of cardiovascular regulation. Although the CVLM and the rVRG are not identical, the CVLM overlaps with a portion of the rVRG. The cardiovascular-related neurons in CVLM are usually ventral to the respiratory neurons recorded in the VRG (Verberne et al., 1999; Mandel and Schreihofer, 2006). A group of neurons in the CVLM are GABAergic (GAD-67 mRNA) and provide the critical link in the baroreceptor reflex between the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM) (Schreihofer and Guyenet, 2003). Inhibition of the CVLM increases the sympathetic nerve response to CO_2 (Moreira et al., 2006). These baro-activated neurons have recently been described to have various types of inspiratory-modulated activity as evident in averaged extracellular recording of neuronal activity triggered by the onset of the phrenic nerve discharge (PND) and their phenotype determined as GABAergic with juxtacellular labeling followed by GAD-67 ISH (Mandel and Schreihofer, 2006).

2.3 PreBötzinger Complex

The preBötzinger Complex (preBötC) is located in the ventrolateral medulla, just ventral to the compact portion of the nucleus ambiguus and extending caudally to where the lateral reticular nucleus divides. These neurons are essential for the generation of the mammalian respiratory rhythm (Smith et al., 1991; Rekling and Feldman, 1998; Feldman et al., 2003; Ramirez and Viemari, 2005; Feldman and Del Negro, 2006; Koizumi et al., 2008). Many of the neurons in the preBötC express the neurokinin-1 receptor (NK1-R) immunoreactivity (Gray et al., 1999, 2001; Guyenet and Wang, 2001; Liu et al., 2001, 2005; Alheid et al., 2002; Thoby-Brisson et al., 2003; Dehkordi et al., 2004) and have a glutamatergic phenotype, i.e. they co-express VGlut2 (Wang et al., 2001; Guyenet et al., 2002). Some of the NK1-R neurons in this region also express mu-opiate receptors (Gray et al., 1999), nitric-oxide synthase (Liu et al., 2001) and a variety of neurotransmitter receptors besides NK1 (Liu et al., 2001). Most of the NK1-R neurons within the preBötC do not co-express GlyT2, choline acetyltransferase, GAD-67 or tyrosine hydroxylase (Wang et al., 2001). A subpopulation of the glutamatergic NK1-R neurons co-express somatostatin and have propriobulbar but not bulbospinal projections (Stornetta et al., 2003a). While all these described small NK1-R neurons belong to the category of propriobulbar expiratory-inspiratory neurons that are a component of the rhythm generating circuit in the preBötC, there are other populations of NK1-R neurons in this area. Directly adjacent in the rostral most part of the rVRG and sometimes overlapping the preBötC are large I-AUG neurons with direct projections to the phrenic motor nucleus. A fraction of these neurons are also NK1-R immunoreactive (Makeham et al., 2001; Guyenet et al., 2002) and glutamatergic (Guyenet et al., 2002) and some co-express pre-pro-enkephalin (Stornetta et al., 2003b). This data reinforces the report by Gray et al. (1999) that the population of NK1-R neurons in the preBötC /rVRG area is heterogeneous.

There are other cell types within the preBötC, not quite so widely studied as the small NK1-R neurons, including e.g., non-NK1-R neurons with either early or late inspiratory activity (Hayes and Del Negro, 2007) and respiratory neurons whose activity is modified by BDNF through the tyrosine kinase B (TrkB) receptor (Thoby-Brisson et al., 2003). A variety of neuronal phenotypes have also been identified by strictly anatomical methods (no evidence that they are respiratory other than location within the preBötC) that contain a variety of substances and receptors, either with or without NK1-R immunoreactivity (Liu et al., 2001; Liu and Wong-Riley, 2002; Liu et al., 2002, 2004, 2005).

2.4 Bötzinger complex

The Bötzinger complex, the rostral extension of the rVRG from the compact portion of the nucleus ambiguus to just behind the facial motor nucleus, contains neurons with several different respiratory-related firing patterns. The neurons with expiratory-related firing have been well-characterized as inhibitory, first by Merrill et al. (Merrill et al., 1983) who used spike-triggered averaging to show that Bötzinger neurons projected monosynaptically to and inhibited dorsal respiratory group (DRG) inspiratory neurons. Other studies followed using similar techniques to show Bötzinger expiratory neurons elicited monosynaptic inhibitory post-synaptic potentials (IPSPs) in other respiratory area neurons including the inspiratory neurons in the caudal and rostral VRG (Fedorko et al., 1989; Jiang and Lipski, 1990), phrenic motoneurons (Merrill and Fedorko, 1984; Tian et al., 1998), laryngeal motoneurons (Ono et al., 2006; Shiba et al., 2007) and in the expiratory neurons in the caudal VRG (Jiang and Lipski, 1990). The major transmitter of these Bötzinger neurons is glycine as demonstrated by a combination of electrophysiological identification, juxtacellular labeling and ISH for both expiratory-augmenting (Schreihofer et al., 1999) and expiratory-decrementing neurons (Ezure et al., 2003). Although the evidence for glycinergic respiratory Bötzinger neurons is convincing, there is some anatomical evidence that neurons in the Bötzinger region projecting to the area of the DRG in NTS are GABAergic (Livingston and Berger, 1989) and that there are a few bulbospinal GABAergic neurons in the region of the Bötzinger complex (Ellenberger, 1999). However these studies were purely anatomical and did not identify the GAD neurons with the physiological characteristics of Bötzinger respiratory neurons.

2.5 Parafacial respiratory group (pFRG)

The pFRG, as described in the neonate with optical or electrophysiological recordings (Onimaru and Homma, 1987. 2003; Tokumasu et al., 2001; Janczewski et al., 2002), has not been well characterized with respect to exact neurotransmitter phenotype, greatly limiting comparison between studies or conducting specific studies with an identified cell type. Although Onimaru and colleagues have evidence for both inhibitory (Arata et al., 1998) and excitatory-type neurons (Janczewski et al., 2002; Onimaru et al., 2006) in this area, they have provided only correlative physiological data rather than direct recording and histochemical identification of pFRG neurons. A recent study by Guyenet and colleagues (Fortuna et al., 2008) hypothesizes that the pFRG in adult might include some of the glycinergic neurons of the Bötzinger complex that develop a pre-I post-I firing pattern when exposed to severe hypoxia. The idea that neurons with a similar pre-I post-I pattern of firing as observed in the neonate correspond to subgroups of expiratory BötC neurons postulated by Ezure (2004) and Fortuna et al. (2008) remains to be confirmed by identification of unique chemical markers that label these neurons both in the neonate and in the adult.

2.6 Retrotrapezoid nucleus (RTN)

The RTN is the most rostral group of neurons in the VRC with an identified phenotype. The discharge of these neurons is weakly modulated by inhibitory feedback from the central pattern generator (CPG). The RTN neurons are chemosensitive and participate at least in the respiratory component of the chemoreflex (i.e. increased respiration in response to carbon dioxide and to afferents from peripheral chemoreceptors signaling hypoxia) (Mulkey et al., 2004; Stornetta et al., 2006; Takakura et al., 2006). These neurons are glutamatergic (i.e. contain VGlut2 mRNA detected by both ISH and RT-PCR) and express the NK1-R (Mulkey et al.,2007a; Dubreuil et al., 2008; Takakura et al., 2008) but are not serotonergic, cholinergic or catecholaminergic. They express the transcription factor Phox2b (Stornetta et al., 2006; Mulkey et al., 2007b); the gene coding for this transcription factor is mutated in congenital central hypoventilation syndrome (CCHS, (Gozal, 1998; Amiel et al., 2003; Weese-Mayer et al., 2003, 2005; Trang et al., 2004; Matera et al., 2004; Trochet et al., 2005; Gaultier et al.,

2005; Bachetti et al., 2005; Todd et al., 2006; Antic et al., 2006; Dubreuil et al., 2008)). Partial loss of this RTN Phox2b neuronal population results in a significant increase in the CO2 threshold for activation of the phrenic nerve under anesthesia (Takakura et al., 2008). Replication in mice of one of the most common mutations observed in humans with CCHS leads a 70% loss of this presumed chemoreceptor population and in turn leads to loss of chemoreflexes and severe hypoventilation in the newborn mice (Dubreuil et al., 2008).

2.7 Dorsal respiratory group (DRG)

Inspiratory neurons in the dorsomedial medulla in and around the NTS have been identified in both cat (Berger et al., 1984) and rat (de Castro et al., 1994), although differences may exist in the projection patterns of these neurons between species. There are several different categories of respiratory-related neurons in the area of the DRG that are distinguished topographically, as well as by their different inputs and firing patterns. Neurons firing synchronously with lung inflation were originally called pump (P) cells (Berger, 1977; Saether et al., 1987) and are unnecessary for the generation of the basic respiratory rhythm. Pump cells receive pulmonary afferents from slowly adapting stretch receptors (SARs) and are responsible for the Hering-Breuer reflex (for reviews see (Coleridge and Coleridge, 1994; Kubin et al., 2006)). The pump cells appear to be mainly inhibitory (Ezure et al., 2002; Tanaka et al., 2003), although there may be an excitatory sub-population not yet definitively characterized (Ezure and Tanaka, 2004). These neurons are located in the ventrolateral NTS in the interstitial subnucleus and slightly rostral to the obex. Their inhibitory nature was confirmed by the observation that they express GAD-67 mRNA and some also co-express GlyT2 mRNA (Ezure and Tanaka, 2004; Takakura et al., 2007). Ezure (2004) suggests that this sub-population of pump cells could co-release GABA and glycine; however, there is no direct evidence of this.

The cells that receive inputs from the rapidly adapting lung stretch receptors (RARs) are quite different from those receiving inputs from SARs. RAR relay neurons are located primarily in the commissural nucleus of the NTS (Lipski et al., 1991; Ezure and Tanaka, 2004) and facilitate inspiratory-related activity. They appear to be neither GAD-67 nor GlyT2-containing and are consequently postulated to be glutamatergic (Ezure and Tanaka, 2004).

Neurons also located caudally within the NTS, mainly within the commissural nucleus, receive inputs from the carotid body chemoreceptors (Finley and Katz, 1992) responding mainly to arterial hypoxia, and project directly (or via higher order relays) to the VRC and to the RTN (Otake et al., 1993; Takakura et al., 2006). The VRC/RTN-projecting neurons are excitatory, i.e. contain VGlut2 mRNA (Takakura et al., 2006), and express the transcription factor Phox 2b (Stornetta et al., 2006).

Cohen and colleagues (Cohen et al., 1974; Cohen and Feldman, 1984) suggested that the DRG inspiratory neurons (i.e. not the pump cells) with direct input from the CPG monosynaptically excited phrenic motor neurons. Lipski and coworkers (Lipski et al., 1983; Duffin and Lipski, 1987) demonstrated that the DRG inspiratory neurons are excitatory using spike-triggered averaging of fast EPSPs in phrenic motoneurons, using action potentials of inspiratory ventrolateral NTS neurons as triggers. There has been no confirmation to date that the excitatory neurotransmitter used by these DRG inspiratory neurons is glutamate.

2.8 Dorsolateral pons- parabrachial nucleus, Kölliker Fuse nucleus

This general area has been termed a “pneumotaxic center” and has been implicated in several aspects of respiratory control including switching between respiratory phases (Lumsden, 1923; Bianchi and St John, 1982; St.John, 1986; Jodkowski et al., 1994) as well as coordination of the breathing pattern (Rybak et al., 2004; Smith et al., 2007). The major respiratory-related projections to this area are from the NTS and from the VRC. Some authors have recorded from

identified respiratory neurons in these areas and studied their morphology and projections (Kobayashi et al., 2005; Ezure and Tanaka, 2006; Song et al., 2006). Yokota et al. (2001, 2004, 2007) have data showing glutamatergic neurons in the Kölliker-Fuse nucleus project to rVRG and/or to the phrenic motor nucleus and suggest that these neurons could be respiratory-related, but to date nobody has identified a major transmitter phenotype in physiologically identified cells in the pontine respiratory centers. Since there are several sub-regions of this general area that have different effects on respiration (Chamberlin and Saper, 1994; Chamberlin, 2004) and neurons located throughout the area have different respiratory-related firing patterns, this cries out for more definitive phenotyping for neurotransmitters of identified neurons in this region.

3. Physiological implications of co-localization of neurotransmitters

The finding that some neurotransmitters are co-localized in respiratory-related neurons compels further hypotheses on what the physiological relevance is of this observation. A summary of possible synaptic configurations of multiple transmitters and receptors is elaborated in the review by Davanger (1996). This includes localization of the two transmitters into separate synaptic terminals or co-localization of transmitter in the same synapse but differential post-synaptic localization of receptors, or co-localization of transmitters that have differential effects on the same postsynaptic receptor. Another possibility is differential release of transmitter depending on the properties of the input to the neuron (e.g. high or low firing frequency).

The co-localization of GlyT2 and GAD-67 in brainstem neurons has been noted by several authors (Stornetta et al., 2004; Ezure and Tanaka, 2004; Takakura et al., 2007). The co-localization of these transmitters has also been reported in terminals using electron microscopy (Dumba et al., 1998; Dugue et al., 2005). Jonas and coworkers provided the first evidence that GABA and glycine can be co-released (Jonas et al., 1998). Both these transmitters are active on hypoglossal motor neurons in brainstem (O'Brien and Berger, 1999). GABA and glycine are packaged by the same vesicular transporter (Sagne et al., 1997; McIntire et al., 1997; Chaudhry et al., 1998) and could be co-packaged in the same vesicles (Burger et al., 1991; Wojcik et al., 2006). Although both GABA and glycine receptors are co-localized postsynaptic to terminals that co-express GABA and glycine (Todd et al., 1996), a recent study by Lu et al. (2008) indicates that GABA co-released with glycine in the medial nucleus of the trapezoid body (auditory brainstem) can act directly at glycine receptors to modify their kinetics. Thus both transmitters could work on the same postsynaptic receptor but through different post-synaptic mechanisms. The co-released transmitters also can exhibit specific effects by working at different postsynaptic receptors. Dugue et al. (2005) in their report studying cerebellar synapses suggest that GABA and glycine co-expressed in terminals by the same Golgi cells have differential effects in their contacts on granule cells and unipolar brush cells because these two target cell types differentially express either GABA receptors (in the case of the granule cells) or glycine receptors (associated predominantly with unipolar brush cells). Neither of these reports indicated a differential distribution of GABA and glycine in the presynaptic terminals- both neurotransmitters seemed to be co-localized in the same terminals. Katsurabayashi et al. (2004) found that a single bouton in spinal cord could release GABA only, glycine only or both transmitters together. This study indicates that vesicles in these boutons could contain either GABA or glycine or both together in the same vesicle, thus raising the possibility that GABA, glycine or the combination could be released differentially, although no further experiments were undertaken to verify this idea.

The co-existence of the excitatory neurotransmitter glutamate with the inhibitory neuropeptide enkephalin is puzzling. One possible explanation however, given that peptides are released with higher stimulation frequencies (Lundberg and Hokfelt, 1983; Iverfeldt et al., 1989; Drake

et al., 1994; Vilim et al., 2000), is that when the system is undergoing maximal stimulation, the inhibitory peptide could serve as an autoregulatory inhibitory feedback to bring the system back towards normal firing. There is evidence for this in the hippocampus. Glutamate and dynorphin are co-localized in mossy fibers in the hippocampus and there is evidence that they are co-released (Terrian et al., 1988; Gannon and Terrian, 1991; Xie et al., 1991; Conner-Kerr et al., 1993; Terman et al., 2000)). The dynorphin is released from mossy fibers after high-frequency synaptic activation (Drake et al., 1994) and reduces the excitation of granule cells by presynaptic inhibition of excitatory amino acid release (Simmons et al., 1994).

Another possible function of the inhibitory peptide co-expressed with the excitatory neurotransmitter could be an amplification of the excitatory signal through inhibition of pre- or post-synaptic inhibitory neurons. No evidence exists to date of co-release of neurotransmitters with neuropeptides in identified respiratory neurons and the function of the inhibitory neuropeptides that have been found to be co-localized in respiratory neurons is still unknown. It is certainly possible that the inhibitory neuropeptides co-localized in excitatory neurons are a mechanism of inhibitory feedback to return the system to a more normal firing pattern during times of high stress, i.e. if the inspiratory drive is too high, the somatostatin in the rhythm-generating neurons of the preBötC is released by the higher frequency stimulation and this inhibits the VRC neurons downstream as well as potentially inhibiting other preBötC reciprocally connected neurons. Similarly, the inspiratory-augmenting pre-motor neurons that co-express enkephalin and glutamate, may release enkephalin during a similar high stress situation where the inspiratory drive is too high to maintain and results in a more hyperpolarized phrenic motor neurons that would fire less frequently. This topic is wide open for further research in the field of respiratory regulation.

4. Summary and conclusions

A few groups of neurons in the respiratory centers have been identified in terms of major ionotropic transmitter (see Fig. 1 and Table). These include the cholinergic expiratory and inspiratory motor neurons (not illustrated), the glycinergic expiratory-augmenting neurons of the Bötzinger complex, the GABAergic pump cells of the DRG, the glutamatergic peripheral chemoreceptor relay neurons of the DRG, the glutamatergic inspiratory- augmenting premotor neurons of the rVRG, the glycinergic expiratory-decrementing neurons of the cVRG, rVRG and BötC, the glutamatergic inspiratory neurons of the preBötC, and the glutamatergic chemoreceptor neurons of the RTN. A few of these neuronal groups have been further characterized with co-localization of other markers: glutamatergic inspiratory-augmenting neurons of the rVRG express enkephalin mRNA and NK1-r immunoreactivity, glutamatergic, presumed-inspiratory neurons of the preBötC express somatostatin and NK1-R immunoreactivity, glutamatergic peripheral chemoreceptor relay neurons of the DRG and glutamatergic chemoreceptive neurons of the RTN co-express Phox2b.

The actual co-release of neurotransmitters in respiratory neurons has not been demonstrated and the physiological ramifications of the co expression of these neurotransmitters require much further study.

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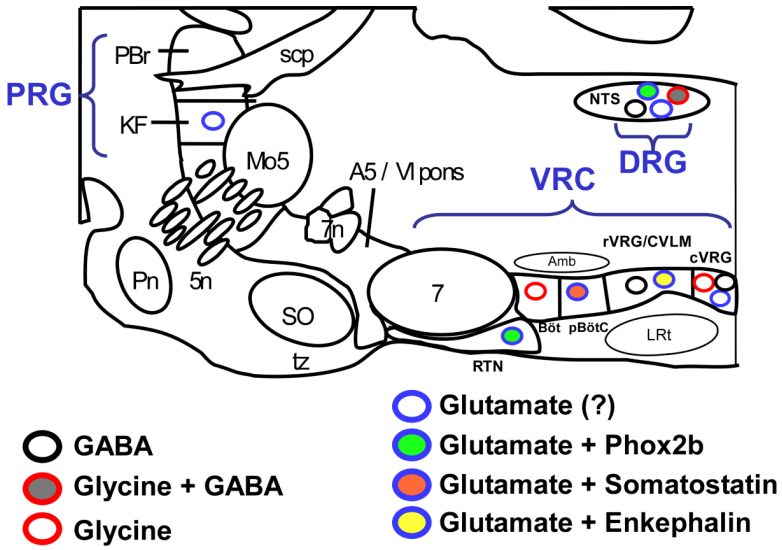

Figure 1.

Summary of respiratory neurons with identified major transmitters (parasagittal section with landmarks after Feldman). Placement of symbols representing phenotypically identified neurons is not meant to be exactly anatomically correct but to denote the region where this cell type is found. Abbreviations: 5n, motor roots of 5th nerve; 7, facial motor nucleus; 7n, facial nerve; Amb, Ambiguus; Böt, Bötzinger area; CVLM, caudal ventrolateral medulla; cVRG, caudal portion of ventral respiratory group; DRG, dorsal respiratory group; KF, Kölliker Fuse nucleus; LRt, lateral reticular nucleus; Mo5, trigeminal motor nucleus; NTS, nucleus of the solitary tract; PBr, Parabrachial nucleus; Pn, Pontine nuclei; PRG, pontine respiratory group; pBötC, PreBötzinger Complex; rVRG, rostral portion of ventral respiratory group; RTN, retrotrapezoid nucleus; scp, superior cerebellar peduncle; VRC, ventral respiratory column

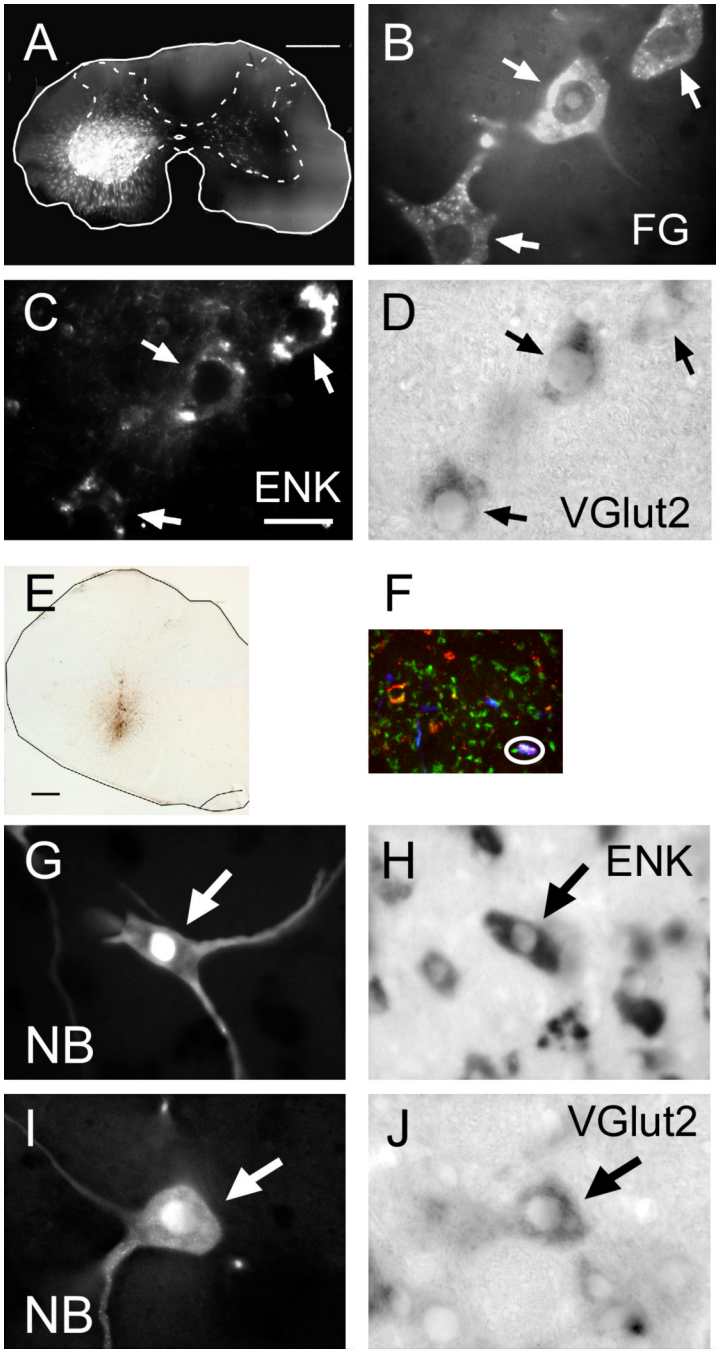

Figure 2.

Example of techniques used to determine multiple transmitters in inspiratory-augmenting neurons of the rVRG. A. FluoroGold injection site in C4 cervical spinal cord. B. Neurons in rVRG retrogradely labeled with FG from injection site in A. C. Same neurons as in B labeled using in situ hybridization with an FITC-tagged riboprobe for pre-proenkephalin (ENK). D. Same neurons as in B labeled with a digoxigenin-tagged riboprobe fir VGlut2. E. BDA injection site in rVRG. F. Circled terminal in ventral horn of cervical spinal cord shows labels for BDA, VGlut2 and enkephalin revealed by immunocytochemistry. G. Inspiratory-augmenting neuron in rVRG labeled with Neurobiotin (NB). H. Same cell as G labeled with riboprobe for ENK mRNA. I. Inspiratory-augmenting neuron in rVRG labeled with

Neurobiotin. J. Same cell as I labeled with riboprobe for VGlut2 mRNA. (Details in (Stornetta et al., 2003)).

Table

Summary of major neurotransmitter phenotypes of identified respiratory neurons

Location	Firing pattern	Neurotransmitter	Co-localized substance
cVRG	E-DEC	acetylcholine (motor neurons) [1]	-
cVRG	E-DEC	glycine [2]	-
cVRG	I-AUG, post-I, E-DEC	GABA [3]	-
cVRG	E-AUG	glutamate? [4]	-
rVRG	I-AUG	glutamate	enkephalin[5], NK1-R[6]
rVRG	E-DEC	glycine [2]	-
preBötC	inspiratory	glutamate	somatostatin[7], NK1-R[6,7]
Bötzinger	E-AUG[8], E-DEC[2]	glycine	-
pFRG	pre-I/post-I	glutamate? [9], glycine? [10]	-
RTN	tonic, CO_2 modulated	glutamate	Phox2b[11], NK1-R[12]
DRG	lung inflation (via SARs)	GABA [13]	glycine[14]
DRG	lung inflation (via RARs)	glutamate? [14]	-
DRG	inspiratory	glutamate? [15]	-
Pons	mixed[16]	glutamate? [17]	-

The firing pattern is in relationship to phrenic nerve discharge in terms of inspiration or expiration or as noted with respect to lung inflation. "?" indicates that the neurotransmitter type has not been confirmed by both physiological and histological methods. Abbreviations: cVRG, caudal respiratory group; DRG, dorsal respiratory group; E-AUG, expiratory augmenting; E-DEC, expiratory decrementing; I-AUG, inspiratory augmenting; I, inspiratory; NK1-R, neurokinin1 receptor; preBötC, preBötzinger complex; RARs, rapidly adapting (lung stretch) receptors; SARs, slowly adapting (lung stretch) receptors.

[1] All respiratory motor neurons are cholinergic. Some are expiratory and some are inspiratory but no additional transmitter has been identified in any of them.

[2] Ezure et al., 2003

[3] Yamazaki et al., 2000. Okazaki et al., 2001. Note that propriobulbar GAD-ir cells with various firing patterns were found throughout the VRC. However, Ezure[2] found the E-DEC cells to be mostly glycinergic. These discrepancies could be due to different methods to identify the cells' phenotypes: Ezure et al. used ISH and the others used GAD immunoreactivity.

[4] Ballantyne and Richter, 1986; Arita et al., 1987; Iscoe, 1998

[5] Stornetta et al., 2003b

[6] Wang et al., 2001; Guyenet et al., 2002

[7] Stornetta et al., 2003a

[8] Schreihofer et al., 1999

[9] Janczewski et al., 2002; Onimaru et al., 2006

[10] Arata et al., 1998; Fortuna et al., 2008

[11] Stornetta et al., 2006; Mulkey et al., 2007b

[12] Mulkey et al., 2007a; Dubreuil et al., 2008; Takakura et al., 2008

[13] Takakura et al., 2007

[14] Ezure and Tanaka, 2004 (~ 26% of GABA DRG neurons also contain glycine)

[15] Lipski et al., 1983; Duffin and Lipski, 1987

[16] Ezure and Tanaka, 2006.

[17] Yokota et al., 2001, 2004, 2007