Published in final edited form as:

J Comp Neurol. 2009 January 20; 512(3): 373–383. doi:10.1002/cne.21897.

GALANIN IS A SELECTIVE MARKER OF THE RETROTRAPEZOID NUCLEUS IN RATS

Ruth L. Stornetta¹, Darko Spirovski², Thiago S. Moreira³, Ana C. Takakura³, Gavin H. West¹, Justin M. Gwilt¹, Paul M. Pilowsky², and Patrice G. Guyenet¹

¹ Department of Pharmacology, University of Virginia, Charlottesville, VA 22908

² Macquarie University, Sydney, 2109 NSW, AUSTRALIA

³ Department of Physiology, UNIFESP-EPM, São Paulo, SP, 04023-060, Brazil

Abstract

The rat retrotrapezoid nucleus (RTN) contains CO₂-activated neurons that contribute to the central chemoreflex and to breathing automaticity. These neurons have two known markers, the transcription factor Phox2b and vesicular glutamate transporter 2 (VGLUT2). Non-catecholaminergic galanin-immunoreactive (ir) neurons within a region of the lower brainstem that seems identical to what is currently defined as the RTN have been previously described. Here we ask whether these galanin-expressing neurons are the same cells as the recently characterized CO₂-sensitive neurons of the RTN.

Using in situ hybridization, we found that pre-pro-galanin (PPGal) mRNA is expressed by an isolated cluster of neurons that is coextensive with the RTN as defined by a population of strongly Phox2b-ir neurons devoid of tyrosine-hydroxylase (Phox2b⁺TH⁻ neurons). This bilateral structure contains about 1000 PPGal-mRNA positive neurons in the rat. The PPGal-mRNA positive neurons were Phox2b⁺TH⁻ and as susceptible to destruction by the toxin [Sar⁹, Met (O₂)¹¹]-substance P as the rest of the RTN Phox2b⁺TH⁻ cells of the RTN. CO₂-activated neurons were recorded in the RTN of anesthetized rats and were labeled with biotinamide. Many of those cells (7/17, 41%, 5 rats) contained PPGal-mRNA.

In conclusion, galanin mRNA is a very specific marker of the glutamatergic Phox2b⁺TH⁻ neurons of the RTN but galanin mRNA identifies only half of these putative central respiratory chemoreceptors.

Keywords

Central chemoreceptors; retrotrapezoid nucleus; galanin; medulla oblongata; Phox2b

INTRODUCTION

In rats, the retrotrapezoid nucleus ((RTN, term originally coined by (Smith et al., 1989)) is a thin sheet of medullary neurons located ventral to the facial motor nucleus (Ellenberger and Feldman, 1990; Cream et al., 2002; Rosin et al., 2006; Stornetta et al., 2006). The RTN provides an excitatory drive to the breathing network and contributes to the central chemoreflex (Nattie, 2001; Li and Nattie, 2002; Feldman et al., 2003; Guyenet, 2008). The dominant neuronal population is glutamatergic and selectively innervates the

pontomedullary regions responsible for respiratory rhythm and pattern generation (Mulkey et al., 2004; Rosin et al., 2006; Stornetta et al., 2006). Their strong activation by hypercapnia in vivo and by acidification in slices indicate that the RTN neurons contribute to central respiratory chemoreception (Ritucci et al., 2005; Guyenet, 2008) but some indirect evidence also suggests that these cells may be involved in respiratory rhythmogenesis, especially during the neonatal period (Onimaru and Homma, 2003; Feldman and Del Negro, 2006; Guyenet, 2008). RTN neurons express high levels of Phox2b (Stornetta et al., 2006; Kang et al., 2007), a homeobox transcription factor whose mutation causes a large decrease in the central chemoreflex and in breathing automaticity during sleep throughout life in man and at birth in mice (Amiel et al., 2003; Dubreuil et al., 2008). This transcription factor is also expressed by catecholaminergic neurons (C1 and A5), visceral and facial motor neurons and by the superior salivatory nucleus (Kang et al., 2007). All these neurons reside in relatively close proximity to the RTN but the putative RTN chemoreceptors express neither tyrosine-hydroxylase nor choline-acetyl transferase and can be distinguished from the surrounding Phox2b-ir neurons on that basis (Kang et al., 2007).

Several groups have noted the presence of galanin-expressing neurons at the ventral surface of the rostral medulla oblongata of the rat or mouse (Melandner et al., 1986a; 1986b; Krukoff et al., 1992; Palkovits and Horvath, 1994). These anatomical studies, especially the detailed distribution of galanin-like immunoreactivity by Melander et al. (1986a; 1986b), identified an isolated group of non-catecholaminergic but galanin-immunoreactive neurons in a region that seems to match the current definition of the RTN (Cream et al., 2002; Stornetta et al., 2006). Because the ventral surface of the medulla oblongata had been implicated in CO₂ sensitivity since the 1960s (Loeschcke, 1982), Krukoff et al. (1992) and Palkovits and Horvath (1994) proposed that these superficial galanin-immunoreactive neurons might be central chemoreceptors. The present study was designed to test this hypothesis.

Here we show that the galanin-expressing neurons of the RTN region have the same general anatomical distribution and histological markers as the previously characterized chemoreceptors (Phox2b, VGLUT2 and absence of TH). We also demonstrate that the galanin-expressing neurons are destroyed by a saporin-containing toxin previously shown to kill the Phox2b-ir neurons of the RTN with notable selectivity (Takakura et al., 2008). Finally, we demonstrate that a large fraction (7/17, 41%) of the CO₂-activated neurons located in the RTN express preprogalanin (PPGal) mRNA. We conclude that galanin is a highly specific marker of a subset of RTN chemoreceptors.

MATERIALS AND METHODS

Whole animal physiology and anatomical experiments were performed in adult male Sprague-Dawley rats (250–350 g; Taconic, Germantown, NY). The University of Virginia's Animal Care and Use Committee approved all animal procedures and protocols.

Perfusions

Rats were deeply anesthetized with pentobarbital, injected with heparin (500 units, intracardially) and perfused through the ascending aorta with 150 ml of phosphate buffered saline (pH 7.4) followed by 4% phosphate-buffered (0.1 M; pH 7.4) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA). Brains were removed and stored in the same fixative overnight at 4° C. Series of coronal sections (30 µm) from the midbrain, pons and medulla were cut at room temperature using a vibrating microtome and stored in cryoprotectant solution at –20° C for up to 2 weeks (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) prior to histological processing. Brain sections prepared in this way were used for all subsequent procedures.

Histology

Immunocytochemical detection of Phox2b and tyrosine hydroxylase (TH) in combination with in situ hybridization for PPGal mRNA—

DNA template for the PPGal riboprobe was amplified by PCR with whole rat brain cDNA using forward primer 5' ggatccatttaggtgacactatagaagctggacggagacacttgga and reverse primer 5' gaattctaatacgactcactatagggagagtgtggccttgaggagtgg which include the SP6 and T7 promoter regions respectively. Antisense cRNA was transcribed using the T7 promoter in the presence of either Digoxigenin-labeled UTP or fluorescein-12-UTP (Roche Applied Science, Indianapolis, IN) to yield an approximately 700 bp riboprobe. VGLUT2 riboprobe was constructed as previously described to yield a 3.3 kb product (Weston et al., 2004). The PPGal sense probe was generated by transcribing the template in the opposite direction using the SP6 polymerase.

Free floating coronal brain sections were first incubated in pre-hybridization solution made as described previously in detail (Stornetta and Guyenet, 1999), then PPGal riboprobe was added and sections were hybridized overnight at 56° C, then rinsed through a series of decreasing salt concentrations and RNase, and subjected to a high temperature rinse at 56° C in the lowest salt concentration. Sections were then blocked with 10% normal horse serum and 0.1% Triton X-100 in Tris-buffered saline (TBS, pH 7.4) for 1–3 hours. Sections were then incubated simultaneously in antibodies for digoxigenin (1:1000, anti-sheep tagged with alkaline phosphatase, Roche), TH (1:2000, anti-mouse, Chemicon, Temecula, CA) and Phox2b (1:800, anti-rabbit, gift from J.-F. Brunet) for 24–72 hours at 4°C.

Sections were then rinsed and reacted with color substrates (BCIP and nitro blue tetrazolium) for alkaline phosphatase and then incubated with secondary antibodies to reveal the TH (goat anti-mouse Alexa488, 1:200, Invitrogen, Carlsbad, CA) and Phox2b (donkey anti-rabbit Cy3, 1:200, Jackson, West Grove, PA). Sections were then rinsed, mounted on gelatin coated slides and covered with Vectashield, an aqueous mounting medium (Vector Laboratories, Burlingame, CA) followed by a coverslip.

Antibody characterizations—Phox2b rabbit polyclonal antibody was raised against the fourteen amino acid C-terminal sequence of the Phox2b protein, a sequence identical in rats and mice, with the addition of a tyrosine at the N-terminal end (YFHRKPGPALKTNLF) (Pattyn et al., 1997). The specificity was originally ascertained by the perfect match between the expression patterns detected by immunohistochemistry and in situ hybridization at pre-natal stages (Pattyn et al., 1997) and this specificity was confirmed by the complete absence of immunoreactivity in *Phox2b* knock-out mice (A. Pattyn and J.-F. Brunet unpublished data). In adult rat brains (present experiments), the C-terminal tetradecapeptide eliminated Phox2b-like immunoreactivity at 3.7 µg / ml.

Mouse anti-TH monoclonal antibody (MAB318; lot number 050910596; Chemicon) was raised against tyrosine hydroxylase purified from PC12 cells and recognizes an epitope on the outside of the regulatory N-terminus of tyrosine hydroxylase. As reported by Chemicon, this antibody recognizes a protein of approximately 59–63 kDa by Western blot and does not cross-react with dopamine-beta-hydroxylase, phenylalanine hydroxylase, tryptophan hydroxylase or phenyl ethanolamine-N-methyl transferase on Western Blots. The pattern of labeling produced in the current study is seen in cell soma and dendrites as well as putative terminals and was restricted to known catecholamine cell groups.

Double in situ hybridization—Detection of PPGal and VGLUT2 mRNAs was performed as described previously for simultaneous detection of two mRNAs (Stornetta et al., 2002; Stornetta et al., 2005) using digoxigenin-11-UTP for the VGLUT2 riboprobe and fluorescein-12-UTP for the PPGal riboprobe. Briefly, free floating sections were treated as

described above for the pre-hybridization and hybridization steps. After the high temperature, low salt rinse at 56° C, sections were rinsed in 0.1M Tris buffered saline pH 7.4 (TBS), then incubated in a solution of 3% H₂O₂ (Sigma) in TBS. Sections were rinsed and blocked for non-specific immunoreactivity by 30 minute incubation with the blocking reagent supplied with the tyramide amplification kit (0.5% in TBS; Perkin Elmer Life Sciences, Boston, MA) and then incubated with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (1:1000, Roche) and a sheep anti-fluorescein antibody conjugated to horseradish peroxidase (1:1000, Roche) in the same blocking solution for 24–48 hours at 4° C. The sections were rinsed in TBS and incubated in tyramide Cy3 (1:75, Perkin Elmer) for 10–12 minutes to detect PPGal mRNA. Sections were then processed for detection of the VGLUT2 mRNA as described above for detection of digoxigenin-labeled riboprobe. Sections were mounted onto slides, air dried and covered with Vectashield as described above.

Mapping and photomicrography—Sections were examined and photographed with a Zeiss Axioskop II microscope (Carl Zeiss Microimaging, Thornwood, NY.) Sections were mapped using the NeuroLucida system (MicroBrightField, Williston, VT) as previously described (Weston et al., 2004). Only cell profiles which contained an obvious nuclear profile were plotted and counted. Alexa 488 fluorescence was observed and photographed using an excitation filter at 480 nm and an emission filter at 535 nm. Cy3 fluorescence was observed and photographed using an excitation filter at 545 nm and an emission filter at 610 nm.

Photographs were taken with a 12-bit color CCD camera (Regina 1300; resolution 1392 X 1042 pixels, Q Imaging, Burnaby, B.C., Canada) and the resulting TIFF files were imported into Adobe Photoshop (Version 7; Adobe Systems, Mountain View, CA). Output levels were adjusted to include all information-containing pixels. Balance and contrast was adjusted to reflect true rendering as much as possible. No other “photo-retouching” was done. Figures were assembled and labeled using Canvas software (Version 9; ACD Systems, Miami, FL).

Single-unit recording and juxtacellular labeling of RTN neurons—These experiments were done in bilaterally vagotomized, artificially ventilated rats prepared as previously described (Mulkey et al., 2004; Guyenet et al., 2005). Briefly, general anesthesia was induced with 5% halothane in 100% O₂. Artificial ventilation with 1.4–1.5% halothane in 100% O₂ was maintained throughout surgery. The surgical procedures (bilateral vagotomy, bladder catheterization, arterial cannulation, phrenic nerve dissection, dorsal transcerebellar access to the ventrolateral medulla oblongata) were standard (Mulkey et al., 2004; Guyenet et al., 2005). After surgery, halothane was reduced to 1%, in 99% oxygen. Muscle relaxation was performed with pancuronium (initial dose: 1 mg/kg i.v.). Rectal temperature was maintained at 37°C, and end-tidal CO₂ was monitored throughout the experiment with a microcapnometer (Columbus Instruments, Ohio, USA). The adequacy of anesthesia was continually monitored by testing for lack of arterial pressure (AP) change and lack of change in phrenic nerve discharge (PND) rate or amplitude to firm toe or tail pinch. At the end of the physiological experiment the rats were deeply anesthetized with halothane (4% until AP reached 40 mmHg) and perfused through the left cardiac ventricle with phosphate-buffered saline (pH 7.4; 150 ml) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4, 500 ml). The brains were post-fixed overnight in the paraformaldehyde solution. The brains were then sectioned in the coronal plane (30 µm) and the sections were kept in cryoprotectant awaiting histological procedures.

Arterial blood pressure, PND, tracheal CO₂ and tracheal pressure were recorded as previously described (Mulkey et al., 2004; Guyenet et al., 2005). RTN neurons were

characterized as described previously (Mulkey et al., 2004; Guyenet et al., 2005). These neurons were found from 150 to 300 μm below the lower edge of the facial motor nucleus and from 200 μm caudal to 300 μm rostral to the caudal boundary of this nucleus (Mulkey et al., 2004; Guyenet et al., 2005). This region lies between coronal planes Bregma -11.8 and -11.3 mm of the Paxinos and Watson (2005) and contains the greatest concentration of chemoreceptors (Stornetta et al., 2006). Besides this specific location, the defining properties of RTN neurons are a strong activation by hypercapnia (discharge threshold at 4–4.5 % CO_2 and firing rate of 6–14 Hz at 10% CO_2), a lack of respiratory modulation at low levels of CO_2 and a modest respiratory entrainment at high levels of CO_2 (Mulkey et al., 2004; Guyenet et al., 2005). Before searching for RTN neurons, ventilation was adjusted to lower end-expiratory CO_2 to 4% at steady-state (60–80 cycles/s; tidal volume 1–1.2 ml/100 g). Variable amounts of pure CO_2 were then added to the breathing mixture to adjust end-expiratory CO_2 to the desired level without changing ventilation parameters.

All analog data (end-expiratory CO_2 , PND, unit activity, AP) were stored on a microcomputer via a micro-1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed off-line using version 5 of the Spike 2 software (CED) as described before (Mulkey et al., 2004; Guyenet et al., 2005). Processing included action potential discrimination and neuronal discharge rate measurement (spikes / sec). PND “integration” ($\int \text{Phr}$) consisted of full-wave rectification and smoothing (τ : 0.015s). Single unit recordings were made using glass pipettes containing 1.5% biotinamide (Molecular Probes, Eugene, OR) in 0.5M sodium acetate (24–30 MegaOhm DC resistance). Juxtacellular labeling of RTN units with biotinamide was done using 2–6 nA 200 ms long pulses of positive current delivered at 2.5Hz as described before (Mulkey et al., 2004; Guyenet et al., 2005).

Injections of toxin into the retrotrapezoid nucleus

The histological material described in the paragraph of the results entitled “RTN PPGal mRNA-positive neurons are destroyed by SSP-SAP” comes from a subset of animals described in a previous publication (Takakura et al., 2008). The toxin ([Sar⁹, Met (O₂)¹¹]-substance P, SSP-SAP, Advanced Targeting Systems, San Diego, CA) was administered while the rats were anesthetized with a mixture of ketamine (75 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg) administered i.m.. Surgery was performed using standard aseptic methods, and after surgery, the rats were treated with the antibiotic ampicillin (100 mg kg^{-1}) and the analgesic ketorolac (0.6 mg kg^{-1} , s.c.). The saporin conjugate was administered by pressure injection using glass pipettes with an external tip diameter of 25 μm . The experimental rats ($N = 10$) received two injections of 30 nl per side, each containing 0.6 ng of toxin. The first injection was placed 200 μm below the lower edge of the facial motor nucleus, 1.6 to 1.9 mm lateral to the midline and 200 μm rostral to the caudal end of the facial motor nucleus. The second injection was placed 400 μm rostral to the first one, also 200 μm below the facial motor nucleus. The injections were made under electrophysiological guidance using a previously described method (Takakura et al., 2008). This protocol was selected because it destroys the Phox2b-expressing neurons of RTN while exerting minimal damage on the nearby catecholaminergic neurons and the facial motor nucleus (Takakura et al., 2008). The animals survived for 2 weeks and were then subjected to electrophysiological experiments under chloralose-urethane anesthesia. As reported before, the toxin produced no observable behavioral effect nor any discernable respiratory distress when the rats were unanesthetized but, under anesthesia, the CO_2 threshold to elicit phrenic nerve activity was very elevated in the toxin-treated rats (Takakura et al., 2008). At the end of the experiment the rats were killed by an overdose of anesthetic followed by intravascular perfusion with saline and formaldehyde as indicated above. Control rats ($N = 6$) received either bilateral injections of physiological saline ($N = 4$) or a single unilateral

injection of 0.15ng of the toxin (N = 2), a dose of toxin previously shown to have no effect (Takakura et al., 2008). These animals were subjected to the same physiological experiments as the rats with SSP-SAP lesions.

RESULTS

The RTN contains a PPGal-positive cell group

PPGal mRNA was detected exactly where Melander et al. (1986a; 1986b) found galanin-immunoreactive neurons in colchicine-treated rats. In the pons, this mRNA was present only in the locus coeruleus, the parabrachial region and the cochlear nucleus. In the medulla oblongata, PPGal mRNA was detected in the nucleus of the solitary tract, especially caudal to the area postrema, in a restricted region of the ventrolateral medulla corresponding to the rostral ventral respiratory group (Alheid et al., 2002; Stornetta et al., 2003b), and, more caudally, in the A1 group of noradrenergic neurons (results not illustrated). PPGal mRNA was barely if at all detectable in the raphe (obscurus, pallidus and parapyramidal) in histological specimens derived from rats that had been killed by formaldehyde perfusion immediately after being anesthetized. A moderate level of mRNA was detected in all three raphe structures in three animals that had been kept under anesthesia for 6–8 hours for electrophysiological experiments. These raphe regions were also among those found by Melander et al. (1986a; 1986b) to contain some galanin immunoreactivity.

Finally, we also confirm that the rostral ventrolateral medulla contains a distinctive and very well-demarcated group of galanin-expressing neurons centered 1.9 mm lateral to the midline and located in close proximity to the ventral surface (Fig. 1). This cell group, henceforth called the RTN PPGal-positive cell group, contained notably higher levels of PPGal mRNA reaction product than the other groups of galanin-expressing neurons identified in the pontomedullary region. RTN PPGal-positive neurons extend from the level of the rostral tip of the inferior olive (400 microns caudal to the end of the facial motor nucleus; level –12.0 mm after Paxinos and Watson) to the caudal end of the trapezoid body (level –10.3 mm after Paxinos and Watson; Fig. 1D). The PPGal mRNA-positive cell group is largest in its caudal region i.e. within 200 µm of a coronal plane that intersects the last facial motor neurons ((Bregma –11.4 to –11.8 mm after (Paxinos and Watson, 2005); Fig. 1B)). Rostral to that level, a very low density of PPGal-positive neurons is present under the entire extent of the facial motor nucleus (Fig. 1C). At the rostral end of the facial motor nucleus, at a level that corresponds to the caudal edge of the trapezoid body (Bregma –10.3mm after (Paxinos and Watson, 2005), the PPGal-positive cluster becomes slightly enlarged. A substantial fraction of the RTN PPGal-positive cell group resides within the marginal layer of the ventral medullary surface (Fig. 1B,C). The rostrocaudal distribution of the RTN PPGal-positive cell group is described quantitatively below.

The ISH protocol performed with the sense probe produced no signal where adjacent sections from the same animal run with the same solutions simultaneously (with the exception of using the anti-sense probe during the hybridization step) produced the usual pattern of PPGal labeling (data not shown).

The RTN PPGal-positive cell group expresses Phox2b and VGLUT2

RTN CO₂-sensitive neurons are non-catecholaminergic (TH-negative) and non-cholinergic but they express both Phox2b and VGLUT2 (Mulkey et al., 2004; Stornetta et al., 2006). The next experiments were designed to test whether the PPGal-positive cell group of the RTN expresses the same markers as the putative chemoreceptors.

In the first series of experiments, coronal sections were reacted for simultaneous detection of TH-immunoreactivity, Phox2b and PPGal mRNA (3 brains; 1 in 3 series of 30 µm sections

extending throughout the rostrocaudal extent of the galanin-positive group). Within the region of interest, TH and PPGal mRNA were never colocalized (0 TH-ir neurons out of 1224 PPGal mRNA-positive neurons counted; Fig. 2A,B). At all levels of the nucleus, every PPGal-positive neuron had a Phox2b-ir nucleus (Fig. 2C,D). The converse was not true in that a substantial proportion of the Phox2b-ir neurons did not contain PPGal mRNA reaction product (Fig. 2C,D). Some of the Phox2b-ir neurons devoid of PPGal mRNA were TH-ir, as expected (Stornetta et al., 2006), but a large percentage were TH-negative. As shown in Figure 3A, the neurons that were positive for both PPGal mRNA and Phox2b were located ventrolateral to the cells that were positive for both TH and Phox2b (the C1 neurons). The Phox2b-ir neurons that were devoid of both TH immunoreactivity and PPGal-mRNA were intermingled with the PPGal-positive cells but more frequently found at the dorsal edge of the PPGal-positive cell group. Although facial motor neurons are Phox2b⁺TH⁻ cells, they can be easily distinguished from the RTN neurons because of their location, much larger and rounder nuclei and considerably lower Phox2b immunoreactivity (Takakura et al., 2008). Facial motor neurons were not counted as Phox2b⁺TH⁻ cells in the present experiments. The graph shown in Figure 3B depicts the rostrocaudal distribution of the three cell groups of interest. Overall, the total number of PPGal-expressing neurons counted in the one-in-three series of 23 sections was 408 ± 23 (3 rats). The number of Phox2b-ir neurons without PPGal mRNA nor TH counted in the same region and in the same series of sections was 408 ± 6 .

The Abercrombie correction (Abercrombie, 1946) was used to obtain an estimate of the total number of PPGal-expressing neurons of the RTN. Since PPGal-positive cells with a visible nucleus were selectively counted, the correction factor was based on the average diameter of the nucleus in the coronal plane ($7 \pm 0.2 \mu\text{m}$) and the section thickness measured at $28.4 \pm 0.5 \mu\text{m}$ after processing (10 sections measured). The nucleus appears oval with the long axis roughly parallel with the base of the brain; therefore we assumed that the height would be the same as the length of the shorter axis in the coronal plane. Based on these values, the total calculated number of PPGal-expressing neurons was $323 \times 3 = 969$ neurons per brain ($N=3$). The total calculated number of Phox2b-positive neurons devoid of both PPGal and TH present in the same region was the same since the number of counted cells was, coincidentally, identical for both groups and we used the same correction factor. We used the same correction factor because cell counting was based on the identification of a labeled nucleus in both cases and the size and shape of the nuclei of the Phox2b-positive neurons devoid of both PPGal and TH was identical to that of the Phox2b-positive neurons with PPGal.

The second experiment was designed to test whether the PPGal-positive neurons possess the other known marker of RTN chemoreceptors, namely VGLUT2 mRNA. This fact could be logically inferred from prior evidence that Phox2b immunoreactivity and VGLUT2 mRNA are virtually entirely co-localized in the RTN but we sought direct evidence. Double in situ hybridization for VGLUT2 and PPGal mRNA (3 coronal sections sampled bilaterally through the RTN region from 3 rats) revealed that the two markers were substantially co-localized (Fig. 2 E,F). However, the VGLUT2 mRNA signal was weaker in this double ISH material than when VGLUT2 was detected on its own. Therefore, because a significant number of false negative observations were expected, we did not perform a quantitative study of the overlap between the two markers.

RTN PPGal mRNA-positive neurons are a subset of RTN chemoreceptors

The previous experiments showed that the PPGal mRNA cell group is coextensive with the RTN and expresses VGLUT2 and Phox2b as do the neurons previously identified as central chemoreceptors. The next experiments were designed to record from the putative chemoreceptors in vivo and to test whether they contain PPGal mRNA.

Seventeen RTN CO₂-sensitive neurons were recorded extracellularly and filled with biotinamide in 5 rats. Up to 3 cells (typically one or two) were labeled on each side and, in most animals, recording and labeling was done on both sides of the brain. It was often possible to match an individual unit recording with a particular biotinamide-labeled neuron recovered by histology based on our records of the relative stereotaxic location and depth of the recordings. Figure 4A-G shows a case where a single cell was labeled on the left side of the brain, allowing its anatomical characteristics to be unambiguously matched to its electrophysiological properties. This neuron had the typical characteristics of previously described CO₂-activated RTN neurons (Mulkey et al., 2004; Guyenet et al., 2005) namely a high sensitivity to CO₂, a lower CO₂ threshold than the phrenic motor outflow, and a discharge rate that saturated at high pCO₂ (Fig. 4A). This saturation is attributed to the existence of a negative feedback from the respiratory pattern generator (Guyenet et al., 2005). Also consistent with prior descriptions (Guyenet et al., 2005), this particular neuron had little central respiratory modulation at low levels of CO₂ (Fig. 4B1–B2) and was entrained to the central respiratory pattern generator only at very high levels of CO₂ (Fig. 4C1–C2). The respiratory modulation exhibited by this neuron was a mixture of early-inspiratory and post-inspiratory inhibition. This was one of the most frequently encountered patterns for RTN chemoreceptors in halothane-anesthetized vagotomized rats (Guyenet et al., 2005). Juxtacellular labeling was performed for approximately 8 minutes (Fig. 4A) as shown by the steady entrainment of the neuron to the pulses of positive current delivered through the recording electrode (Fig. 4D). This procedure produced an intense labeling of the soma and of the dendrites that permitted reconstruction of a substantial portion of the neuron (Fig. 4E,F). The cell soma resided in very close proximity (150 microns) to the ventral surface and a substantial portion of the dendrites were spread out within the 50 micron-thick marginal layer of the medulla oblongata in a predominantly coronal orientation similar to previously documented RTN chemoreceptive neurons (Mulkey et al., 2004; Weston et al., 2004). As shown in Fig. 4G, the soma of this neuron contained a high level of PPGal mRNA reaction product.

The electrophysiological characteristics of the other 16 CO₂-activated neurons were very similar to that of the cell discussed above and were within the range of previously published values for this cell group (CO₂ threshold between 4 and 4.5% end-expiratory CO₂, discharge rate between 8–15 Hz at saturation) (Mulkey et al., 2004; Guyenet et al., 2005). Most labeled cells were either lightly labeled with biotinamide or too close to another labeled neuron to permit reliable reconstruction of their dendritic structure. Therefore we plotted the location of the biotinamide cell bodies and determined whether they were positive for PPGal mRNA. Figure 4H shows the results of the seventeen histologically recovered cells. All the labeled neurons were found under the caudal edge of the facial motor nucleus and within 350 microns of the ventral surface, i.e. within the confines of the previously characterized RTN (Stornetta et al., 2006). Seven out of 17 cells (41%), including a neuron that had its soma within the marginal layer (Fig. 4G), were strongly PPGal-positive. On average, the PPGal-negative neurons were located slightly dorsal to their PPGal-positive counterparts (Fig. 4H). This observation agrees with the fact that the non-catecholaminergic Phox2b-expressing cells devoid of PPGal are somewhat more numerous in the dorsal half of the RTN (Figure 3A). Our dorsal approach to the RTN may have introduced a slight sampling bias in favor of the CO₂-activated neurons located at the dorsal edge of RTN. Therefore, the proportion of CO₂-activated RTN neurons that express galanin could be somewhat higher than the 41% figure reported here.

RTN PPGal mRNA-positive neurons are destroyed by SSP-SAP

In a recently published study we demonstrated that the TH-negative Phox2b-ir neurons of the RTN region were destroyed by local microinjection of SSP-SAP ([Sar⁹, Met (O₂)¹¹]-

substance P (Takakura et al., 2008)). Coronal sections from rats that had been used in this prior study were selected to test whether SSP-SAP destroys the galanin-expressing neurons. As shown in Figure 5, the PPGal-positive neurons were destroyed to the same extent as the generic Phox2b⁺TH⁺ neurons regardless of their location within the nucleus. In these experiments, the toxin was injected in the caudal portion of the nucleus and the rostral cluster of Phox2b⁺TH⁺ neurons were essentially unaffected regardless of whether they expressed PPGal (Fig. 5).

DISCUSSION

In this study we demonstrate that the group of galanin-expressing neurons located at the surface of the rostral ventrolateral medulla and caudal pons is a large subset (around 50%) of a previously described group of glutamatergic Phox2b-expressing neurons that have the electrophysiological characteristics of central chemoreceptors.

Location of PPGal mRNA in the lower brainstem: comparison with galanin immunoreactivity

In the pontomedullary region, we detected PPGal mRNA precisely where prior authors have described the presence of galanin immunoreactivity after colchicine treatment (Melander et al., 1986a; Melander et al., 1986b; Krukoff et al., 1992; Palkovits and Horvath, 1994). The similarities with the detailed descriptions of Melander and colleagues (1986a; 1986b) are especially striking. These similarities have been listed in the results section and need not be revisited except in regard to the expression of galanin by the medullary raphe. We did find PPGal mRNA in all the raphe subdivisions where galanin immunoreactivity had been detected following colchicine treatment (Melander et al., 1986a; Melander et al., 1986b). However, PPGal mRNA was virtually undetectable in the medullary raphe of rats that had been killed by an overdose of pentobarbital (brief anesthesia) followed immediately by aldehyde perfusion whereas a large number of moderately labeled neurons could be detected in three rats that had been subjected to a long period of anesthesia for electrophysiological recording of the RTN. Since the labeling intensity in other areas was not different and the same galanin probe was used in these experiments, the discrepancy suggests that, in the raphe, galanin expression may be rapidly induced by anesthesia, surgery or unknown factors associated with the experimentation under anesthesia. This interpretation is based at present on a limited number of rats and needs to be verified by a larger study. However, the fact that PPGal mRNA synthesis or accumulation can be rapidly increased is already well established. For example colchicine treatment and peripheral nerve injury (Hokfelt, 2005) as well as Fluorogold injection (Finley et al., 1995) are instances where rapid accumulation of galanin mRNA occurs.

We confirm the existence of a very superficial group of galanin-expressing neurons that extends about 1.7 mm rostrocaudally at the surface of the ventrolateral medulla from the level of the trapezoid body to that of the Böttinger area of the ventral respiratory column (for anatomical definitions see (Alheid et al., 2002)). Most of this galanin-expressing cell group except its caudal end is located below the facial motor nucleus and its cells express a particularly high level of the PPGal message. We also confirm that these cells are not catecholaminergic and that they reside ventrolateral and rostral to the C1 group of adrenergic neurons with limited intermingling of their cell bodies (Melander et al., 1986b). The galanin-ir neurons located in the RTN region were found to be non-serotonergic (Melander et al., 1986b). This conclusion is supported by our observation that the PPGal-positive neurons express Phox2b and VGLUT2, markers that are absent from pontomedullary serotonergic neurons (Mulkey et al., 2004; Stornetta et al., 2006). The cluster of PPGal-positive neurons is in good register with the originally proposed nuclear boundaries of the RTN (Ellenberger and Feldman, 1990; Cream et al., 2002). The PPGal-

positive cluster extends a full 1.7 mm from the caudal end of the trapezoid body to the level of the Bötzing subdivision of the ventral respiratory column (−10.3 to −12 mm caudal to Bregma). The region encompassed by the PPGal-positive cluster can also be identified by the presence of intensely Phox2b-ir and VGLUT2 mRNA-positive neurons already proven to be activated by CO₂ in vivo and by acidification in vitro (Stornetta et al., 2006; Mulkey et al., 2007). This area of the brain is well demarcated from the more medial parapyramidal region which is characterized by the presence of intensely NK1R-ir neurons, the lateral B3 group of serotonergic neurons and small-sized cholinergic neurons (Ruggiero et al., 1990; Mulkey et al., 2004; Nattie and Li, 2006). Although this more medial region has been included in the RTN in the past (Nattie and Li, 2002), there is little evidence at the cellular level that it is involved in central chemoreflexes and its cytology is very different (Takakura et al., 2008). None of the Phox2b⁺ TH[−] neurons of the RTN and immediately surrounding region project to the spinal cord (Kang et al., 2007). Since the PPGal-mRNA positive neurons are a subset of Phox2b⁺ TH[−] neurons, they are not bulbospinal and, therefore, must be distinct from the non-catecholaminergic presympathetic neurons that reside nearby (Schreihofer et al., 2000; Guyenet, 2006).

Based on the Abercrombie correction (Abercrombie, 1946), we estimate that the RTN contains a total of 993 PPGal-positive neurons. The Abercrombie method has many biases (Williams and Rakic, 1988) but its theoretical error rate is relatively low (<2%) if section thickness exceeds the greatest nuclear height in the vertical plane by a factor of 1.5 (Clarke, 1992). These conditions are probably met in our material given the size of the nucleus measured in the coronal plane and the section thickness.

PPGal mRNA identifies a subset of RTN chemoreceptors

The region encompassed by the PPGal-positive cells corresponds well to the range of sites where focal CO₂ dialysis stimulates breathing (Li et al., 1999) and where various types of lesions attenuate the central chemoreflex (Nattie and Li, 2000; Nattie and Li, 2002; Takakura et al., 2008). This region of the brain contains neurons that are strongly activated by CO₂ in vivo and by bath acidification in slices (Mulkey et al., 2007). The pH-sensitive neurons recorded in vitro have the same distinctive morphology and express the same sets of markers as the CO₂-activated neurons, namely VGLUT2, Phox2b, no TH (Mulkey et al., 2007) (Mulkey et al., 2004; Stornetta et al., 2006). For these and many other reasons, the Phox2b⁺/TH[−] neurons of the RTN are considered to be central respiratory chemoreceptors (Guyenet, 2008).

The present study demonstrates that the PPGal-positive neurons of the RTN region have the same general location and the same markers as the putative chemoreceptors (VGLUT2, Phox2b and no TH). In addition, we provide direct evidence that a large fraction of the CO₂-activated neurons of RTN contain PPGal mRNA (41%). This percentage is reasonably close to the fraction of the Phox2b⁺/TH[−] neurons of the RTN region that contains PPGal mRNA. The slight discrepancy (41% vs. 50%) could be due to chance given the relatively limited number of neurons that were studied (N=17). This discrepancy could also be due to a sampling bias that favored recording from the CO₂-sensitive cells that are devoid of PPGal because these cells reside slightly dorsal to the PPGal-positive neurons on average.

Based on this evidence we propose that the PPGal-containing neurons of the RTN region represent a subset of the Phox2b- and VGLUT2-positive central chemoreceptors. Collectively, this cell group innervates the ventral respiratory column, the parabrachial nuclei and the ventrolateral nucleus of the solitary tract (Rosin et al., 2006). We have not tested whether the galanin-expressing subset of RTN chemoreceptors innervates all of these areas but we can infer that these cells innervate the parabrachial nuclei since Krukoff et al. (1992) found that galanin-ir neurons located in the RTN region are retrogradely labeled from

the dorsal pons. We also presume that the galanin-expressing cells innervate the ventral respiratory column because the vast majority of the CO₂-activated neurons of the RTN can be antidromically activated from this region (Mulkey et al., 2004). Consistent with this supposition, the region of the VRG contains a moderate density of galanin-ir terminals but these terminals could also derive from galanin-expressing neurons located in the nucleus of the solitary tract (Melander et al., 1986a; Melander et al., 1986b).

In the neonate rat brain, the RTN or a very closely related region contains pre-inspiratory neurons a.k.a. the parafacial respiratory group (pFRG) (Onimaru and Homma, 2003). These neurons may be part of an oscillator implicated in the generation of inspiration (Onimaru and Homma, 2003) or expiration (Feldman and Del Negro, 2006). The pFRG neurons of the neonate have not been characterized from a biochemical standpoint and their relationship to the Phox2b⁺TH⁺ neurons discussed here is unknown. Under abnormal conditions (combination of high CO₂ and hypoxia), the RTN region of the adult anesthetized rat contains neurons that develop a pre-inspiratory post-inspiratory discharge pattern similar to the pFRG but these cells are the glycinergic Bötzing neurons, not the Phox2b⁺TH⁺ neurons of RTN (Fortuna et al., 2008). Although we show here that the RTN contains at least two different neurochemical varieties of neurons, one with and the other without galanin, neither subset displays a pFRG-type discharge under our normal experimental conditions *in vivo*.

Role of galanin released by RTN neurons

Galanin signals through three subtypes of G-protein coupled receptors (Hokfelt, 2005). The acute administration of galanin typically inhibits neuronal activity and reduces transmitter release but excitatory effects mediated via a decrease in potassium conductance have also been occasionally reported (Xu et al., 2005). Galanin synthesis is highly inducible and probably plays some role in long-term neuronal responses to injury (Hokfelt, 2005).

VGLUT2 gives neurons the ability to release glutamate by exocytosis (Freneau et al., 2004) therefore RTN neurons, which express this protein (Mulkey et al., 2004), are very probably glutamatergic and excitatory. The fact that these neurons synthesize the generally inhibitory peptide galanin is not incompatible with this view because, in the lower brainstem, inhibitory peptides are frequently synthesized by glutamatergic neurons. Examples include the enkephalin-expressing inspiratory premotor neurons of the rostral VRG and the somatostatin-expressing respiratory rhythm-generating neurons of the pre-Bötzing complex (McCrimmon et al., 1989; Stornetta et al., 2003a; Stornetta et al., 2003b; Tan et al., 2008). These neurons are undoubtedly glutamatergic but opioid and somatostatin agonists inhibit respiratory neurons and breathing when they are injected into the lower brainstem (Kalia et al., 1984; Bianchi et al., 1995; Llona and Eugenin, 2005). Classic neurotrophic factors like BDNF can also produce acute changes in neuronal electrical activity (Kafitz et al., 1999). Whether galanin released by RTN neurons is involved in short-term synaptic transmission or mediates some form of neuronal plasticity remains to be determined.

Conclusions

The previously identified cluster of galanin-expressing neurons located at the ventral surface of the rostral medulla is part of and coextensive with the RTN. PPgal mRNA is a highly specific single marker of the RTN but it is only expressed by around 50% of the total population of the RTN neurons with putative central chemoreceptor function.

Acknowledgments

This work was supported by grants from the National Institutes of Health to P.G.G (HL74011 and HL 28785).

LITERATURE CITED

- Abercrombie M. Estimation of nuclear population from microtome sections. *Anat Rec.* 1946; 94:239–247. [PubMed: 21015608]
- Alheid GF, Gray PA, Jiang MC, Feldman JL, McCrimmon DR. Parvalbumin in respiratory neurons of the ventrolateral medulla of the adult rat. *J Neurocytol.* 2002; 31:693–717. [PubMed: 14501208]
- Amiel J, Laudier B, Attie-Bitach T, Trang H, de PL, Gener B, Trochet D, Etchevers H, Ray P, Simonneau M, Vekemans M, Munnich A, Gaultier C, Lyonnet S. Polyalanine expansion and frameshift mutations of the paired-like homeobox gene PHOX2B in congenital central hypoventilation syndrome. *Nat Genet.* 2003; 33:459–461. [PubMed: 12640453]
- Bianchi AL, Denavit-Saubie M, Champagnat J. Central control of breathing in mammals: neuronal circuitry, membrane properties, and neurotransmitters. *Physiol Rev.* 1995; 75:1–45. [PubMed: 7831394]
- Clarke PGH. How inaccurate is the Abercrombie correction factor for cell counts. *Trends Neurosci.* 1992; 15(6):211. [PubMed: 1378664]
- Cream C, Li A, Nattie E. The retrotrapezoid nucleus (RTN): local cytoarchitecture and afferent connections. *Respir Physiol Neurobiol.* 2002; 130:121–137.
- Dubreuil V, Ramanantsoa N, Trochet D, Vaubourg V, Amiel J, Gallego J, Brunet JF, Goridis C. A human mutation in Phox2b causes lack of CO₂ chemosensitivity, fatal central apnoea and specific loss of parafacial neurons. *Proc Natl Acad Sci.* 2008; 105:1067–1072. [PubMed: 18198276]
- Ellenberger HH, Feldman JL. Brainstem connections of the rostral ventral respiratory group of the rat. *Brain Res.* 1990; 513:35–42. [PubMed: 2350683]
- Feldman JL, Del Negro CA. Looking for inspiration: new perspectives on respiratory rhythm. *Nat Rev Neurosci.* 2006; 7:232–242. [PubMed: 16495944]
- Feldman JL, Mitchell GS, Nattie EE. Breathing: Rhythmicity, Plasticity, Chemosensitivity. *Annu Rev Neurosci.* 2003; 26:239–266. [PubMed: 12598679]
- Finley JC, Erickson JT, Katz DM. Galanin expression in carotid body afferent neurons. *Neurosci.* 1995; 68:937–942.
- Fortuna MG, West GH, Stornetta RL, Guyenet PG. Botzinger expiratory-augmenting neurons and the parafacial respiratory group. *J Neurosci.* 2008; 28:2506–2515. [PubMed: 18322095]
- Fremeau RT, Voglmaier S, Seal RP, Edwards RH. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci.* 2004; 27:98–103. [PubMed: 15102489]
- Guyenet PG. The sympathetic control of blood pressure. *Nat Rev Neurosci.* 2006; 7:335–346. [PubMed: 16760914]
- Guyenet PG. The 2008 Carl Ludwig Lecture: retrotrapezoid nucleus, CO₂ homeostasis, and breathing automaticity. *J Appl Physiol.* 2008; 105:404–416. [PubMed: 18535135]
- Guyenet PG, Mulkey DK, Stornetta RL, Bayliss DA. Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. *J Neurosci.* 2005; 25:8938–8947. [PubMed: 16192384]
- Hokfelt T. Galanin and its receptors: introduction to the Third International Symposium, San Diego, California, USA, 21–22 October 2004. *Neuropeptides.* 2005; 39:125–142. [PubMed: 15908000]
- Kafitz KW, Rose CR, Thoenen H, Konnerth A. Neurotrophin-evoked rapid excitation through TrkB receptors. *Nature.* 1999; 401:918–921. [PubMed: 10553907]
- Kalia M, Fuxe K, Agnati LF, Hokfelt T, Harfstrand A. Somatostatin produces apnea and is localized in medullary respiratory nuclei: a possible role in apneic syndromes. *Brain Res.* 1984; 296:339–344. [PubMed: 6142757]
- Kang BJ, Chang DA, Mackay DD, West GH, Moreira TS, Takakura AC, Gwilt JM, Guyenet PG, Stornetta RL. Central nervous system distribution of the transcription factor Phox2b in the adult rat. *J Comp Neurol.* 2007; 503:627–641. [PubMed: 17559094]
- Krukoff TL, Vu T, Harris KH, Aippersbach S, Jhamandas JH. Neurons in the rat medulla-oblongata containing neuropeptide-Y-like, angiotensin-II-like, or galanin-like immunoreactivity project to the parabrachial nucleus. *Neurosci.* 1992; 47(1):175–184.
- Li A, Nattie E. CO₂ dialysis in one chemoreceptor site, the RTN: stimulus intensity and sensitivity in the awake rat. *Respir Physiol Neurobiol.* 2002; 133:11–22. [PubMed: 12385727]

- Li A, Randall M, Nattie EE. CO(2) microdialysis in retrotrapezoid nucleus of the rat increases breathing in wakefulness but not in sleep. *J Appl Physiol.* 1999; 87:910–919. [PubMed: 10484557]
- Llona I, Eugenin J. Central actions of somatostatin in the generation and control of breathing. *Biol Res.* 2005; 38:347–352. [PubMed: 16579516]
- Loeschcke HH. Central chemosensitivity and the reaction theory. *J Physiol.* 1982; 332:1–24. [PubMed: 6818338]
- McCrimmon DR, Smith JC, Feldman JL. Involvement of excitatory amino acids in neurotransmission of inspiratory drive to spinal respiratory motoneurons. *J Neurosci.* 1989; 9:1910–1921. [PubMed: 2542482]
- Melander T, Hokfelt T, Rokaeus A. Distribution of galaninlike immunoreactivity in the rat central nervous system. *J Comp Neurol.* 1986a; 248:475–517. [PubMed: 2424949]
- Melander T, Hokfelt T, Rokaeus A, Cuello AC, Oertel WH, Verhofstad A, Goldstein M. Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. *J Neurosci.* 1986b; 6:3640–3654. [PubMed: 2432203]
- Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA, Guyenet PG. Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci.* 2004; 7:1360–1369. [PubMed: 15558061]
- Mulkey DK, Talley EM, Stornetta RL, Siegel AR, West GH, Chen X, Sen N, Mistry AM, Guyenet PG, Bayliss DA. TASK channels determine pH sensitivity in select respiratory neurons but do not contribute to central respiratory chemosensitivity. *J Neurosci.* 2007; 27:14049–14058. [PubMed: 18094244]
- Nattie E, Li A. Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. *J Appl Physiol.* 2000; 89:153–162. [PubMed: 10904047]
- Nattie E, Li A. Neurokinin-1 receptor-expressing neurons in the ventral medulla are essential for normal central and peripheral chemoreception in the conscious rat. *J Appl Physiol.* 2006; 101:1596–1606. [PubMed: 16902062]
- Nattie EE. Chemoreception and tonic drive in the retrotrapezoid nucleus (RTN) region of the awake rat: bicuculline and muscimol dialysis in the RTN. *Adv Exp Med Biol.* 2001; 499:27–32. [PubMed: 11729890]
- Nattie EE, Li A. Substance P-saporin lesion of neurons with NK1 receptors in one chemoreceptor site in rats decreases ventilation and chemosensitivity. *J Physiol.* 2002; 544:603–616. [PubMed: 12381830]
- Onimaru H, Homma I. A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci.* 2003; 23:1478–1486. [PubMed: 12598636]
- Palkovits M, Horvath S. Galanin immunoreactive neurons in the medulla oblongata of rats. *Acta Biol Hung.* 1994; 45:399–417. [PubMed: 7537001]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates* 3921. San Diego: Elsevier Academic Press; 2005.
- Ritucci NA, Erlichman JS, Leiter JC, Putnam RW. Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus. *Am J Physiol Regul Integr Comp Physiol.* 2005; 289:R851–R861. [PubMed: 15905224]
- Rosin DL, Chang DA, Guyenet PG. Afferent and efferent connections of the rat retrotrapezoid nucleus. *J Comp Neurol.* 2006; 499:64–89. [PubMed: 16958085]
- Ruggiero DA, Giuliano R, Anwar M, Stornetta R, Reis DJ. Anatomical substrates of cholinergic-autonomic regulation in the rat. *J Comp Neurol.* 1990; 292:1–53. [PubMed: 2312784]
- Schreihofer AM, Stornetta RL, Guyenet PG. Regulation of sympathetic tone and arterial pressure by rostral ventrolateral medulla after depletion of C1 cells in rat. *J Physiol.* 2000; 221–236. [PubMed: 11080264]
- Smith JC, Morrison DE, Ellenberger HH, Otto MR, Feldman JL. Brainstem projections to the major respiratory neuron populations in the medulla of the cat. *J Comp Neurol.* 1989; 281:69–96. [PubMed: 2466879]
- Stornetta RL, Guyenet PG. Distribution of glutamic acid decarboxylase mRNA-containing neurons in rat medulla projecting to thoracic spinal cord in relation to monoaminergic brainstem neurons. *J Comp Neurol.* 1999; 407:367–380. [PubMed: 10320217]

- Stornetta RL, Moreira TS, Takakura AC, Kang BJ, Chang DA, West GH, Brunet JF, Mulkey DK, Bayliss DA, Guyenet PG. Expression of Phox2b by brainstem neurons involved in chemosensory integration in the adult rat. *J Neurosci*. 2006; 26:10305–10314. [PubMed: 17021186]
- Stornetta RL, Rosin DL, Simmons JR, McQuiston TJ, Vujovic N, Weston MC, Guyenet PG. Coexpression of vesicular glutamate transporter-3 and gamma-aminobutyric acidergic markers in rat rostral medullary raphe and intermediolateral cell column. *J Comp Neurol*. 2005; 492:477–494. [PubMed: 16228993]
- Stornetta RL, Rosin DL, Wang H, Sevigny CP, Weston MC, Guyenet PG. A group of glutamatergic interneurons expressing high levels of both neurokinin-1 receptors and somatostatin identifies the region of the pre-Botzinger complex. *J Comp Neurol*. 2003a; 455:499–512. [PubMed: 12508323]
- Stornetta RL, Sevigny CP, Guyenet PG. Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. *J Comp Neurol*. 2002; 444:191–206. [PubMed: 11840474]
- Stornetta RL, Sevigny CP, Guyenet PG. Inspiratory augmenting bulbospinal neurons express both Glutamatergic and enkephalinergic phenotypes. *J Comp Neurol*. 2003b; 455:113–124. [PubMed: 12455000]
- Takakura AC, Moreira TS, Stornetta RL, West GH, Gwilt JM, Guyenet PG. Selective lesions of retrotrapezoid Phox2b-expressing neurons raises the apneic threshold in rats. *J Physiol*. 2008 In press.
- Tan W, Janczewski WA, Yang P, Shao XM, Callaway EM, Feldman JL. Silencing preBotzinger Complex somatostatin-expressing neurons induces persistent apnea in awake rat. *Nat Neurosci*. 2008; 11:538–540. [PubMed: 18391943]
- Weston MC, Stornetta RL, Guyenet PG. Glutamatergic neuronal projections from the marginal layer of the rostral ventral medulla to the respiratory centers in rats. *J Comp Neurol*. 2004; 473:73–85. [PubMed: 15067719]
- Williams RW, Rakic P. Three-dimensional counting: an accurate and direct method to estimate numbers of cells in sectioned material. *J Comp Neurol*. 1988; 278:344–352. [PubMed: 3216047]
- Xu ZQ, Zheng K, Hokfelt T. Electrophysiological studies on galanin effects in brain--progress during the last six years. *Neuropeptides*. 2005; 39:269–275. [PubMed: 15944021]

Figure 1. Pre-pro-Galanin (PPGal) mRNA distribution throughout the retrotrapezoid nucleus (RTN)

A. Bregma level -12.0 , note the cluster of PPGal mRNA neurons in the ventrolateral medulla. B. Bregma level -11.7 , the bulk of the PPGal mRNA neurons are located near the ventral surface in the ventrolateral medulla overlapping with the RTN region. C. Bregma level -11.5 , the PPGal mRNA neurons hug the ventral surface below the facial motor nucleus (7). D. Bregma level -10.1 , the rostral extent of the PPGal mRNA neurons in the RTN at the caudal extension of the trapezoid body (tz) lateral to the caudal end of the superior olive (SO). Other abbreviations: Amb, ambiguus nucleus; IO, inferior olive; py, pyramidal tract. Scale bar in D = 250 microns. Bregma levels after the atlas of Paxinos and Watson (2005).

Figure 2. Galanin-containing neurons co-express Phox2b and VGlut2 but are not catecholaminergic

A. PPGal mRNA containing neuron adjacent to B. catecholaminergic neurons revealed with tyrosine hydroxylase (TH) immunoreactivity. C. PPGal mRNA neurons in the RTN co-localize with D. Phox2b immunoreactive (ir) neurons. Arrows point to Phox2b-ir cells that do not contain PPGal mRNA. E. PPGal mRNA neurons revealed with a FITC-labeled riboprobe are co-localized with F. VGlut2 mRNA containing neurons revealed with a digoxigenin-labeled riboprobe. A–B, C–D and E–F are pairs of photomicrographs of the same section taken with either brightfield or fluorescent light. Scale bar in F = 50 microns.

Figure 3. Distribution of PPGal mRNA, Phox2b immunoreactivity and TH immunoreactivity in coronal sections of rat brain through the RTN

Bregma levels (after Paxinos and Watson (2005)) are indicated by the negative numbers below each section. Cells were counted in an area bounded by the end of the spinal trigeminal tract (sp5) laterally and either the ventral edge of the ambiguous nucleus (in more caudal sections) or the facial motor nucleus. The graph represents the distribution of the different phenotypic classes of neurons counted in 3 rats. The mean number of cells for a particular Bregma level is plotted as the solid line with error bars indicated by the vertical lines. PPGal mRNA plus Phox2b immunoreactive (ir) neurons are indicated by blue stars in the coronal sections and by the blue diamonds and line in the graph. Phox2b-ir neurons that do not contain TH ir are indicated by the green circles in the coronal sections and the green line in the graph. TH-ir neurons that were not Phox2b-ir or PPGal mRNA positive are represented by red squares in the coronal sections and by the red squares and line in the graph.

Figure 4. RTN chemoreceptors contain PPGal mRNA

A. Identification of a chemosensitive neuron. This cell had a CO₂ threshold 1.2 % lower than the PND and close to 4%. B1–B2: at low levels of CO₂ (at the time indicated by the arrowhead above the letter B in panel A), this neuron display a tonic discharge with merely a trace of central respiratory modulation revealed in B2 by PND-triggered averaging. C1–C2: at very high level of CO₂ (10%, at the time indicated by the arrowhead above the letter C in panel A), this neuron displays a modest reduction in discharge probability during the post-inspiratory period (C1 original traces; C2 PND-triggered average of the unit discharge rate). D: excerpt from the period during which the cell was labeled juxtacellularly (period indicated by the arrowhead above the letter D in panel A). E: location of the cell represented in panels A–D. F: reconstruction and projection in the coronal plane of the same cell. G: high power photograph of the cell soma (scale 20 microns) showing its high content of PPGal mRNA. H: computer-assisted plot of the location of the 17 biotinamide-labeled chemosensitive neurons (scale: 1 mm). All the recorded cells were within 200 microns of the coronal plane represented in the figure (−11.5 mm after Paxinos and Watson). The filled squares are the PPGal-positive neurons, the open circles are the PPGal-negative neurons. Cells that were recorded on the right side of the brain are shown on a section that was flipped for convenience of presentation. I: RTN PPGal-positive neuron that was recorded in the marginal layer (scale: 40 microns)

Figure 5. RTN PPGal-positive neurons are destroyed by SSP-SAP

A. Rostrocaudal distribution of the Phox2b⁺TH⁻ neurons of the RTN in 6 control rats (filled circles) and in 10 experimental rats (open circles) subjected to two injections of 0.6ng SSP-SAP in the caudal half of the RTN. The ordinate represents uncorrected numbers of Phox2b-ir nuclear profiles detected per section on both sides of a one-in-three series of 30 μ m-thick sections. Section alignment between rats was done by assigning the last section containing facial motor neurons the level of 11.6 mm caudal to Bregma for ease of comparison with the atlas of Paxinos and Watson (Paxinos and Watson, 2005). B. Rostrocaudal distribution of the PPGal mRNA-containing neurons detected in an alternate series of sections from the same two groups of rats. The ordinate represents uncorrected numbers of PPGal mRNA-

containing somatic profiles detected per section within the same region of the ventrolateral medulla.