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CO₂-inhibited neurons in the medullary raphe are GABAergic

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Abstract

Previous studies have reported subsets of medullary raphe neurons that are either stimulated or inhibited by CO₂/pH *in vitro*, *in situ*, and *in vivo*. We tested the hypothesis that medullary raphe CO₂-inhibited neurons are GABAergic. Extracellular recordings in unanesthetized juvenile *in situ* rat preparations showed reversible hypercapnia-induced suppression of 19% (63/323) of medullary raphe neurons, and this suppression persisted after antagonism of NMDA, AMPA/kainate, and GABA_A receptors. We stained a subset of CO₂-inhibited cells and found that most (11/12) had glutamic acid decarboxylase 67 immunoreactivity (GAD67-ir). These data indicate that the majority of acidosis-inhibited medullary raphe neurons are GABAergic, and that their chemosensitivity is independent of major fast synaptic inputs. Thus, CO₂-sensitive GABAergic neurons may play a role in central CO₂/pH chemoreception.

Keywords

raphe; breathing; chemosensitivity; GABA

1. Introduction

Subsets of specialized cells within the brainstem are sensitive to changes in CO₂/pH (central CO₂ chemoreceptors). CO₂-dependent changes in the activity of these cells are thought to critically contribute to arterial blood gas homeostasis by driving acute adjustments in ventilation and/or perfusion. Several studies have suggested a major role for serotonergic (5-HT) neurons in the medullary raphe nuclei in the CO₂ chemoreflex, including cellular

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recordings *in vitro* (Richerson et al., 1995; Wang et al., 1998, 1999) and *in situ* (Iceman et al., 2013), as well as lesion (Da Silva et al., 2011; Dias et al., 2007; Dreshaj et al., 1998; Nattie et al., 2004) and focal acidosis studies *in vivo* (Bernard et al., 1996; Hodges et al., 2004a; b; Nattie and Li, 2001). Some medullary raphé neurons that are stimulated by hypercapnic acidosis stain positively for markers of 5-HT neurons, and possess the increased firing rates induced by hypercapnia in the presence of inhibitors of fast synaptic transmission (Wang et al., 2001). These and other data suggest that a subset of raphé 5-HT neurons are intrinsically CO₂-sensitive and modulate hypercapnic responses *in vivo*.

Investigations attempting to determine the location and function of central respiratory chemoreceptors have focused primarily on neurons that increase their activity in response to hypercapnic acidosis. However, it is equally likely that neuronal inhibition/disinhibition may play a role in chemoreception (Nuding et al., 2009). For example, hypercapnia hyperpolarizes a subset of medullary neurons in organotypic cultures (Wellner-Kienitz and Shams, 1998) and CO₂ decreases discharge frequency of CO₂-sensitive GABAergic neurons in medullary slices (Kuribayashi et al., 2008). About 15% of raphé neurons in acute slice are CO₂-inhibited (Richerson, 1995; Wang and Richerson, 1999) and, in primary culture, up to 27% of raphé neurons are inhibited by CO₂ (Wang et al., 1998). These acidosis-inhibited cells do not contain 5-HT (Wang et al., 2001), and a preliminary report demonstrates that they are GABAergic (Hodges et al., 2005).

Given these data, it has been postulated that CO₂-stimulated raphé 5-HT neurons largely excite respiratory neurons, and that a second effect of hypercapnia in the raphé is to suppress tonic raphé inhibition of the respiratory network. This could occur through direct, CO₂-mediated inhibition of inhibitory raphé neurons, which produces an additive “push-pull” enhancement of ventilation through both excitation and disinhibition (Richerson et al., 2001).

The purpose of this study was to determine: 1) if CO₂-inhibited neurons are present in the medullary raphé in a relatively intact preparation, 2) if they are GABAergic, and 3) if their response to CO₂ is independent of fast synaptic inputs.

2. Methods

2.1. Animals and surgery

All experiments were done in accordance with the guidelines of the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health and were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee. Experiments were conducted in preparations derived from juvenile (P20–P30; 60–150g) male Simonsen albino rats; (n=105; Sprague-Dawley derived; Simonsen Laboratories) *in situ* using the perfused decerebrate juvenile rat brainstem preparation, as per published methods (Corcoran et al., 2013; Mosher et al., 2014). Briefly, animals were heparinized (0.3 mL of 1,000 i.u./mL; i.p.; Baxter, Deerfield, IL), then deeply anesthetized with isoflurane. Preparations were transected below the diaphragm, immersed in ice-chilled perfusate, and decerebrated rostral to the superior colliculi. Subsequent procedures were conducted in the absence of anesthesia as decerebration renders animals insensitive to pain. Each preparation was placed

prone in a stereotaxic head frame, and the descending aorta was cannulated retrogradely with a double-lumen catheter and perfused with solution at a temperature of 31°C. The perfusate contained the following (in mM): MgSO₄ (1.0); NaH₂PO₄ (1.25); KCl (4.0); NaHCO₃ (24); NaCl (115); CaCl₂ (2.0); D-glucose (10); Ficoll 70 (0.18). Under baseline conditions, perfusing solutions were equilibrated with 95% O₂ - 5% CO₂ (PCO₂ 33 mmHg; pH 7.4). Wilson et al. (2001) previously demonstrated that these procedures led to brainstem tissue pH that matched that of the perfusate. The neuromuscular blocker gallamine triethiodide (60 mg/L) was added to the perfusate to eliminate movement. The pressure of aortic perfusion (measured with a blood pressure transducer attached to the second lumen) was increased gradually to 50–75 mmHg and then held constant. Perfusate was collected and recirculated. The levels of O₂ and CO₂ in the perfusate were maintained by equilibrating a perfusate reservoir with gas mixtures produced with a precision gas mixer (GSM-2, CWE) and verified with a CO₂ analyzer (CD-3A, Applied Electrochemistry). Lacking hemoglobin, solution hyperoxia (PO₂ of approximately 600 mmHg) is necessary to maintain O₂ content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all conditions. Baseline perfusate conditions approximated normocapnic plasma *in vivo*. Hypercapnic challenges (91% O₂ - 9% CO₂; PCO₂ 60 mmHg; pH 7.2) produced conditions comparable to the maximal respiratory acidosis induced by a 4% elevation in inspired CO₂.

2.2. Extracellular recordings

Extracellular recordings of medullary raphé neurons were made using pulled glass capillary electrodes (15–40 MΩ), filled with biotinamide hydrobromide (Life Technologies) dissolved at 5% in 0.5 M sodium acetate. We targeted regions of the medullary raphé (including the raphé obscurus, raphé magnus, and raphé pallidus) along the midline (0–0.1 mm lateral) between 0 and 3.25 mm caudal to the interaural line, 10–12 mm below the dorsal surface. These are areas from which CO₂ sensitive neurons have been identified *in vitro*. Electrodes were placed above raphé target areas and driven into the tissue using a fine stepping motor (2 μm steps; Burleigh Inchworm) held in a stereotaxic 5-axes micropositioner integrated with a digital brain atlas (Benchmark Angle Two; MyNeuroLab). Neuronal recordings were always initiated under baseline conditions, followed by brief (5 min) moderate hypercapnic challenges (9% CO₂) before a return to baseline. Baseline firing was recorded for each unit in normocapnia, followed by a 5 min moderate hypercapnic challenge, and then a 5 min minimum normocapnic recovery period. Electrodes were connected to an Axon Multiclamp 700B intracellular amplifier (Molecular Devices) with high pass filter at 300 Hz and low pass filter at 1 kHz Bessel via an Axon CV7B high impedance headstage (Molecular Devices). Signals were digitized using Spike 2 (CED) or LabChart (AD Instruments), sampled (>10 kHz) and stored as computer data files for subsequent analysis.

To assess the network dependence of chemosensitivity, in some cases (n=15) gas challenge protocols were performed after addition to the perfusate of antagonists to block major fast synaptic inputs (“FSI blockade”). The antagonists blocked NMDA- and AMPA/kainite-type glutamate receptors ((3-((±)2-carboxypiperazin-4yl)propyl-1-phosphate; CPP, 10 μM; 6-cyano-7-nitroquinoxaline-2,3-dione; CNQX, 20 μM), glycine (strychnine hemisulfate; 1 μM), and GABA_A (±bicuculline freebase; 20 μM). This combination of drugs blocks

common FSI, thereby limiting network inputs to spontaneously active neurons without affecting their intrinsic properties (Peña et al., 2004).

2.3. Biotinamide fills

Extracellular recordings were made with an intracellular amplifier (Axon Multiclamp 700B) in current clamp mode, so that current could be injected through the electrode while action potentials (extracellular field potentials) were monitored. Neurons were indiscriminately assessed for response to hypercapnia. After the completion of the trial, if the neuron's signal amplitude was sufficiently large, we attempted to individually fill the neuron with biotinamide using the juxtacellular labeling method (Iceman et al., 2013; Iceman and Harris, 2014; Pinault, 1996). Recorded neurons were individually filled with biotinamide by applying positive-current pulses (400 ms duration, 50% duty cycle) of gradually increasing intensity (0–10 nA maximum in 0.2 nA steps) to each cell through the bridge circuit of the recording amplifier until entrainment of cell discharge to the current pulse was achieved. Cell entrainment was maintained for at least 30 s. These current pulses trigger the iontophoretic ejection of biotinamide and entrainment facilitates uptake of this marker by the recorded and entrained cell. Entrainment was never initiated when multiple units were visible, and double neuron or ectopic labeling was not observed. After termination of entrainment, a 30-min period allowed biotinamide to disperse within the neuron before tissue fixation. The stereotaxic coordinates of the recording site were noted. During recording of single cells, fluctuations in spike amplitude were sometimes observed due to slight changes in relative extracellular position of the electrode and had no correlation with gas treatment or cell discharge. Spike height, width, and shape were monitored before, during, and after juxtacellular entrainment to ensure that only one cell was recorded and labeled (Iceman et al., 2013; Iceman and Harris, 2014; Pinault, 1996). Neurons were selected for recording and hypercapnic challenges when we were confident that the electrode tip was located within the medullary raphé, the recorded unit was firing spontaneously, and the unit's signal amplitude was sufficiently large to ensure a satisfactory recording.

2.4. Immunohistochemistry

After juxtacellular labeling, rats were perfused through the descending aorta with fixative, 4% paraformaldehyde in 0.1 M PBS. Brainstems were then removed and stored overnight in the fixative. A series of coronal sections (60 μ m) were cut through the medulla using a Vibratome (Leica Biosystems), and processed free-floating. Biotinamide introduced into single neurons by juxtacellular labeling was revealed with a streptavidin-Alexa 546 conjugate (Life Technologies; S-11225; 4 μ g/mL). Sections were incubated in blocking buffer for 1 h (0.3% Triton X-100, 5% normal goat serum in 0.1 M PBS) then overnight in antibody for the GABA-synthesizing enzyme glutamic acid decarboxylase 67 (GAD67). We used mouse anti-GAD67 monoclonal primary antibody (Millipore MAB5406; 1:500 dilution in blocking buffer) followed by 1 h incubation in a secondary Alexa 488-labeled goat anti-mouse antibody (Life Technologies A-11029; 1:500 dilution in 0.1 M PBS with 5% normal goat serum). Some sections were incubated with sheep anti-tryptophan hydroxylase (TPH) polyclonal antibody (Millipore AB1541; 1:250) in blocking buffer with 0.3% Triton X-100 to reveal immunoreactivity for serotonin synthesis, or in rabbit anti-

GAD65/67 (Millipore AB1511; 1:500), or in mouse anti-GABA (Sigma A2052; 5 µg/mL) to reveal immunoreactivity for GABA synthesis. Immunohistochemical controls included incubation of medullary sections without primary antibody to rule out non-specific binding of the fluorophores and incubation without fluorophores to rule out autofluorescence. Low-magnification (10x) images were used to determine the location of biotinamide-labeled cells in relation to anatomical landmarks (ventral surface, pyramids, etc.); this placement was correlated with areas of the raphé and cells were mapped onto the brain atlas at the appropriate location. Local biotinamide- and GAD67- or TPH-related fluorescence was visualized to identify absence or presence of colocalization of GAD67 or TPH in the soma of biotinamide-labeled neurons. Fluorophores were individually excited and emission spectra were collected separately to minimize interference using a Zeiss LSM510 confocal microscope: biotin-filled neuron, Alexa 546, 543 nm laser, filter BP 560–615; anti-GAD67, Alexa 488, 488 nm laser, filter BP505–530. Images at one focal plane were collected with a 10x objective and z-stacks were collected with a 40x objective. 40x images are presented as a collapsed projection of a z-stack.

2.5. Extracellular recording data analysis

We recorded individual extracellular unit activity using computer spike sorting software (Spike 2, CED; Spike Histogram, AD Instruments). Stable 1- to 3-min periods of single unit firing were analyzed before, during, and after hypercapnic challenge (“baseline”, “hypercapnia”, and “recovery”, respectively) to provide a mean value for unit firing frequency (spikes/s), mean interspike interval (ms), standard deviation and standard error of mean interspike interval, and spike width. If a neuron responded to hypercapnic perfusate with a change in firing frequency greater than 20% relative to baseline and returned toward baseline upon return to normocapnia, the neuron was considered chemosensitive and the recording continued (Wang et al., 1998). We recorded 323 individual cells throughout gas challenges. Accounts of CO₂-stimulated cells have been previously reported (Iceman et al., 2013; Iceman and Harris, 2014) and are not considered here. Statistical differences were calculated using a 2-way repeated measures ANOVA (factorial; one factor repetition) with Holm–Sidak pairwise multiple comparison procedures or a Mann–Whitney Rank Sum test. Overall significance level was set to $p = 0.05$ (SigmaPlot 12). Values are expressed as means $\pm$ standard error of the mean.

3. Results

3.1. Medullary raphé neurons express markers for GABA synthesis

We hypothesized that medullary raphé neurons that are inhibited by hypercapnic acidosis are GABAergic. Thus, we probed juvenile rat brainstem sections with antibodies targeting isoforms of the GABA synthetic enzyme GAD. Figure 1 shows immunohistochemical staining for GAD65, GAD67, GABA, and TPH immunoreactivity. GAD67 immunoreactivity (GAD67-ir) and GABA-ir co-localize in soma of medullary raphé neurons (Fig. 1A). Vesicular GAD65/67-ir closely apposes TPH-ir neurons in the medullary raphé (Fig. 1B). GABAergic soma and terminals are plentifully distributed in the medullary raphé and surround cell bodies and processes of 5-HT neurons.

3.2. The medullary raphe contains neurons that are reversibly inhibited by CO₂

We then performed single unit extracellular recordings of spontaneously firing neurons in the medullary raphe. We indiscriminately selected cells in the medullary raphe to obtain recordings throughout 5 min of baseline normocapnia, 5 min of moderate hypercapnia (9% CO₂), and at least 5 min of recovery (normocapnia). We classified cells as CO₂-inhibited if they decreased firing rate relative to baseline by >20% during hypercapnia, and displayed at least some recovery toward baseline with return to normocapnia (Fig. 2A). Out of 323 recorded neurons, 63 (19%) were CO₂-inhibited.

3.3. CO₂-inhibited raphe cells synthesize GABA, but not 5-HT

After completion of the gas challenge protocols, 16 CO₂-inhibited cells recorded were juxtacellularly filled with biotinamide, subsequently recovered after tissue sectioning, and successfully visualized. We immunostained tissue sections for GAD67-ir (n=12) and/or TPH-ir (n=6). Most of the processed cells were GAD67-ir (11/12). None of the processed cells was TPH-ir (0/6), and therefore not serotonergic. One such non-5-HT CO₂-inhibited cell is illustrated in Figure 2B and shown to be located among neighboring 5-HT neurons. GAD67-ir confirms GABAergic identity of another CO₂-inhibited raphe cell (Fig. 2C).

3.4. CO₂-inhibited raphe neurons are sensitive to hypercapnia under FSI blockade conditions

To assess whether or not the chemosensitivity of CO₂-inhibited raphe neurons was dependent on certain network inputs, we bath applied a cocktail of antagonists designed to block common fast synaptic inputs ("FSI blockade"; Peña et al., 2004). The cocktail contained antagonists for NMDA- and AMPA/kainate-type glutamate receptors (CPP; CNQX), glycine (strychnine), and GABA_A (bicuculline) receptors. Figure 2D illustrates a single raphe neuron that was reversibly inhibited by CO₂. After bath application of FSI blockade, the same neuron was similarly reversibly CO₂-inhibited during a second hypercapnic challenge. This cell was juxtacellularly filled and revealed to be a GABAergic raphe neuron.

We categorized all the CO₂-inhibited cells characterized into those recorded without FSI blockade ("drug-free"; n=63), and those recorded during FSI blockade (n=15). Average firing rates during normocapnia, hypercapnia, and recovery were 4.00 ± 0.62 Hz, 2.62 ± 0.44 Hz, and 3.54 ± 0.53 Hz, respectively, for the drug-free group, and 4.23 ± 2.09 Hz, 2.00 ± 0.72 Hz, and 2.06 ± 0.52 Hz, respectively, for the FSI blockade group (Fig. 3A). Mean firing frequencies differed with gas treatments within both the drug-free and FSI blockade groups (factorial 2-way RMANOVA; gas treatment factor $F_{2,152}=12.792$, $p<0.001$), confirming our designation of these cells as CO₂-inhibited. Mean firing frequencies in both groups were inhibited by hypercapnia (Fig. 3A; *drug-free, $t=4.271$, $p<0.001$; ϕ FSI blockade, $t=3.356$, $p=0.003$). Cells recovered from hypercapnic challenge in drug free conditions, as there was no difference between mean firing rates for normocapnic baseline and normocapnic recovery ($t=1.443$, $p>0.151$). FSI blockade affected recovery from hypercapnia in some cells, but not others, resulting in a mean firing rate during recovery that was significantly different from FSI blockade baseline (ϕ ; $t=3.266$, $p=0.003$). However, comparison of mean baseline, hypercapnic, and recovery firing frequencies between drug-

free and FSI blockade groups indicate no difference between the groups, suggesting that FSI blockade did not affect mean firing frequency during any gas treatment (factorial 2-way RMANOVA; drug treatment factor $F_{1,76}=0.273$, $p=0.603$; baseline, $t=0.178$, $p=0.859$; hypercapnia, $t=0.489$, $p=0.626$; recovery, $t=1.165$, $p=0.247$).

Results of a factorial 2-way RMANOVA indicate no difference in hypercapnic response between the groups, illustrating that FSI blockade did not affect response to CO₂ (no between-factors interaction effect; $F_{2,152}=2.650$, $p=0.074$). Figure 3B shows the firing frequencies expressed proportional to baseline normocapnic firing frequency (0%=baseline). During hypercapnia, CO₂-inhibited cells decreased firing by $41 \pm 2.2\%$ in drug-free conditions, and by $48 \pm 6.3\%$ with FSI blockade. Drug-free CO₂-inhibited cells recovered to a mean firing rate of $108 \pm 8.6\%$ of baseline. With FSI blockade, CO₂-inhibited cells returned to a mean firing rate of $78 \pm 7.8\%$. The lack of differences in proportional hypercapnic responses ($U=419.00$, $p=0.502$) and recoveries ($U=374.00$, $p=0.214$) between drug-free and FSI blockade groups supports the conclusion that, on average, drug-free and FSI blockade cells had similar responses to CO₂.

Under FSI blockade conditions, we found discrepancies in recovery of CO₂-inhibited cells. A few (4/15) cells did not display signs of recovery at all. Two of the 15 cells had an especially dramatic hypercapnic response (nearly ceased firing); these clearly increased firing rate during the recovery period but did not recover completely. The remainder (9/15) returned to baseline firing rates during the recovery period. Because of the fact that the cells in FSI blockade conditions displayed one of these three distinct recovery responses, variability in cell firing after FSI blockade precludes clear interpretation of trends pertinent to recovery from hypercapnia. We observed that CO₂-inhibited cells, in general, required longer normocapnic recovery periods to return to baseline firing rates when compared to CO₂-stimulated cells we have described elsewhere (Iceman et al., 2013; Iceman and Harris, 2014). Some of the CO₂-inhibited cells may have still been in the process of returning to a baseline firing rate when we terminated recordings, and we would have possibly observed a complete recovery had we extended the recording period.

4. Discussion

Maintenance of systemic pH/CO₂ homeostasis is accomplished by acute adjustments in alveolar ventilation and perfusion, and deviations in arterial/cerebrospinal fluid CO₂ drive changes in the activity of central respiratory chemoreceptors. The data we present suggest that raphé GABAergic neurons may contribute to the CO₂ chemoreflex, as proposed by the “push-pull” model of central chemosensitivity influenced by serotonergic and GABAergic raphé neurons (Richerson et al., 2001).

We demonstrate that CO₂-inhibited cells are common in the medullary raphé *in situ* and that a large majority of these cells synthesize GABA, but do not synthesize serotonin. Their hypercapnic sensitivity does not depend on major excitatory (glutamatergic) or inhibitory (glycinergic/GABAergic) fast synaptic inputs, nor is it an artifact of culture, as it occurs in an intact neural network *in situ*. However, FSI blockade did impair recovery from hypercapnia in some cases (Section 3.4), revealing the possibility of network effects on CO₂

sensitivity. Our report of mean firing rate decreases of 41–48% during hypercapnia for CO₂-inhibited cells *in situ* (Fig. 3B) is consistent with previous data obtained in both acute slice and in culture. In acute slice, 15% of raphé neurons are CO₂-inhibited, with a mean decrease in firing rate of 39–67% from baseline with hypercapnia (Richerson, 1995; Wang and Richerson, 1999). Previously, Wang and colleagues (1998; 2001) reported about 27% of raphé neurons in culture were inhibited by CO₂ during hypercapnia, and mean firing rate decreased 44–75% from baseline, with similar changes in pH to those of the present study (pH 7.4 to 7.2). The responses of those medullary raphé acidosis-inhibited neurons also did not depend on major fast synaptic inputs for chemosensitivity, suggesting that they may be intrinsically chemosensitive (as is required for characterization as a chemoreceptor *per se*). Although these studies restricted major fast-synaptic inputs, a number of potential modulatory mechanisms remain untested.

Most of the CO₂-inhibited cells that we juxtacellularly filled and tested for GAD67-ir were immunoreactive (11/12) while all of those tested for TPH-ir were immunonegative (6/6; Fig. 2). These results are consistent with previous reports that 15/19 acidosis-inhibited cultured raphé neurons were GAD67-ir (Hodges et al., 2005), and that 16/16 acidosis-inhibited cultured raphé neurons lacked TPH-ir (Wang et al., 2001). We conclude that most CO₂-inhibited raphé cells are GABAergic. However, because 50% of CO₂-unresponsive raphé neurons are GAD67-ir *in vitro* (Hodges et al., 2005), we do not conclude that all GABAergic raphé neurons are CO₂-inhibited. We observed that soma of CO₂-inhibited cells were generally granular or fusiform in shape, and had smaller diameters and less extensive branching than CO₂-stimulated cells (Iceman et al., 2013; Iceman and Harris, 2014). This is consistent with previous observations in culture (Wang et al., 1998; 2001). Similarly, juxtacellularly characterized raphé GAD67-ir cells *in vivo* have smaller soma diameters than 5-HT cells (Allers and Sharp, 2003).

GABAergic raphé neurons tonically inhibit neighboring raphé 5-HT neurons (Boothman et al., 2006; Challis et al., 2014) through both GABA_A and GABA_B receptors (Abellan et al., 2000; Gallagher and Aghajanian, 1976; Tao et al., 1996), and interconnected raphé 5-HT and GABAergic neurons have been proposed to reciprocally influence each other (Bagdy et al., 2000; Richardson-Jones et al., 2011). Therefore, inhibition of the GABAergic raphé neurons by CO₂ could attenuate GABA release, resulting in disinhibition of postsynaptic targets, including 5-HT cells. The roles of inhibition and disinhibition in ventilatory control are not well-characterized. As GABA is a main inhibitory neurotransmitter, GABAergic mechanisms surely contribute to control of breathing (McCrimmon et al., 1997) and are likely involved in maintenance of blood gas homeostasis. Raphé CO₂-inhibited neurons have been proposed to tonically limit respiratory output, such that under hypercapnic conditions respiratory output is disinhibited (Wang et al., 2001).

Gourine and Spyer (2001) report that disinhibition (removal of GABA_A-mediated inhibition) contributes to the hypercapnic ventilatory response, and that GABA application to inspiratory neurons eliminates the response. Half of GABAergic neurons on the ventral medullary surface (overlapping regions traditionally considered to be chemosensitive) are hyperpolarized by hypercapnia, and GABA_A receptors are expressed by CO₂-sensitive neurons in those regions (Kanazawa et al., 1998; Kuribayashi et al., 2008). GABA_A receptor

activation also interferes with the hypoglossal reflex response to hypercapnia (Liu et al., 2003) and when GABA synthesis is prevented in the hypothalamus, the hypercapnic ventilatory response is enhanced (Peano et al., 1992). This literature supports an important role for brainstem GABA in ventilatory chemoreflexes. Our data further demonstrate that medullary raphé acidosis-inhibited neurons are GABAergic.

GABAergic raphé neurons are also involved in other aspects of homeostatic regulation, including pain responsiveness, temperature, stress, heart rate, arousal, and blood pressure regulation (Cao et al., 2006; Cao and Morrison, 2003; Cerri et al., 2013; Challis et al., 2014; Darnall et al., 2012; DiMicco et al., 2006; Winkler et al., 2006; Zaretsky et al., 2003a; b). Hellman and Mason (2012) propose that raphé magnus collectively integrates inputs and regulates sympathetic/parasympathetic tone, to produce appropriate homeostatic balance. This “gain-setting” is accomplished, in part, through activity of pain-sensing “OFF” cells (93% of which are GABAergic; Winkler et al., 2006). These raphé magnus OFF cells also decrease discharge during spontaneous tachypnea, and when stimulated, produce respiratory depression and exaggerated respiratory sinus arrhythmia indicative of parasympathetic activation (Hellman and Mason, 2012; Hellman et al., 2007; 2009;). Verner and colleagues (2004; 2008) proposed that the dramatic apnea, bradycardia, and hypotension resulting from stimulation of raphé magnus was caused by activation of inhibitory (likely GABA) neurons that project to brain respiratory and cardiovascular sites. Consistent with this concept, when GABA activity is disrupted in the medullary raphé the resulting disinhibition increases sympathetic outflow (Cerri et al., 2010; Morrison, 1999; 2001a; b). The potential role of raphé GABA inhibition of sympathetic response is supported by neuroanatomical studies demonstrating projections of GAD-ir raphé cells to sympathetic spinal cord regions (Antal et al., 1996; Blessing, 1990; Blessing et al., 1987; Jones et al., 1991; Reichling and Basbaum, 1990; Stornetta and Guyenet, 1999; Stornetta et al., 2005; reviewed by Stornetta, 2009). GABAergic raphé neurons may have overlapping responses to multimodal stimuli, consistent with their diverse homeostatic roles.

The anatomical targets and functional roles of acidosis-inhibited GABAergic raphé cells remain to be determined, as do the consequences of their dysfunction. Raphé serotonergic neurons provide well-characterized phrenic motor nucleus innervation (recent review by Hilaire et al., 2010), and 5-HT raphé neurons directly modulate breathing (DePuy et al., 2011). Raphé GABAergic neurons also project to the phrenic motor nucleus in the spinal cord (Cao et al., 2006), however, their influence on breathing remains to be determined. Our results demonstrate that a population of raphé GABAergic neurons are chemosensitive and, with published data, offer a potential role for raphé cells in central CO₂ chemoreception.

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Highlights

The medullary raphé contains neurons that are reversibly inhibited by CO₂

CO₂-inhibited raphé cells synthesize GABA, but not serotonin

Fast synaptic blockade does not impede CO₂ –responsiveness of these neurons

CO₂-inhibited raphé neurons may play a role in central chemoreception

Figure 1. The medullary raphe contains GABAergic neurons

Somatic GAD67 immunoreactivity is present in the medullary raphe of tissue sections from juvenile rats (A), and is co-localized with immunoreactivity for GABA. Vesicular GAD65/67 immunoreactivity (B) is visible in a punctate distribution surrounding cell bodies and neuronal processes, including serotonergic neurons (TPH).

Figure 2. CO₂-inhibited neurons occur in the raphe, and synthesize GABA but do not synthesize serotonin

A) Recordings from a single CO₂-inhibited raphe neuron show spontaneous firing of 0.4 Hz during exposure to 5% CO₂, a 45% decrease in firing frequency (to 0.2 Hz) with exposure to 9% CO₂, and recovery (to 0.5 Hz) with return to normocapnic conditions. (B) A neuron, firing spontaneously at 18.2 Hz, decreased firing to 12.9 Hz, a 29% change during hypercapnia. The recorded cell was juxtacellularly filled with biotinamide (red) and TPH-ir revealed the position of 5-HT neurons (green). A lack of colocalization on the merged image demonstrates that the recorded cell was non-5-HT, within the raphe, and closely apposed to TPH-ir 5-HT neurons. (C) Another spontaneously active neuron (0.4 Hz) decreased firing to 0.2 Hz (a 44% decrease) during hypercapnia. Colocalization between the filled neuron (red) and GAD67-ir (green) identifies it as a GABAergic neuron. (D) Shown is a CO₂-inhibited neuron *in situ* firing at a baseline of 4.5 Hz with 5% CO₂. Its firing decreased by 60% (to 1.8 Hz) with 9% CO₂. Firing frequency recovered (4.0 Hz), and was unchanged by application of FSI blockade (4.1 Hz). Cell firing was decreased again by hypercapnia under continued FSI blockade (2.0 Hz at 9% CO₂) and recovered with a return to 5% CO₂ (3.0 Hz). A coronal section (Paxinos and Watson, 1998) shows the location of the biotinamide filled (red) cell within the raphe magnus (10x). A higher magnification image (40x) revealed a population of GAD67-ir GABAergic cells (green). The recorded cell was GAD67-ir.

Figure 3. CO₂-inhibited neurons in the raphé are similarly chemosensitive under drug-free and FSI blockade conditions

(A) Mean firing frequencies of all CO₂-inhibited raphé neurons recorded without FSI blockade (“drug-free”; n=63; grey bars) decreased (*) during hypercapnia (9% CO₂), and recovered upon return to normocapnia (5% CO₂). Mean firing frequencies of all CO₂-inhibited raphé neurons recorded under conditions of FSI blockade (n=15; white bars) also decreased (ϕ) during hypercapnia. Normocapnic recovery firing rates of FSI blockade cells did not return to baseline levels on average (ϕ). Symbols denote difference from baseline normocapnic values. (B) Shown are hypercapnic and recovery normocapnic firing frequencies expressed proportional to baseline normocapnic firing rates (0% = no change from baseline firing rate) for drug-free (n=63) and FSI blockade (n=15) conditions. Neither hypercapnic nor recovery responses differed between the groups.