HCN channels contribute to serotonergic modulation of ventral surface chemosensitive neurons and respiratory activity

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Hawkins VE, Hawryluk JM, Takakura AC, Tzingounis AV, Moreira TS, Mulkey DK. HCN channels contribute to serotonergic modulation of ventral surface chemosensitive neurons and respiratory activity. J Neurophysiol 113: 1195–1205, 2015. First published November 26, 2014; doi:10.1152/jn.00487.2014.—Chemosensitive neurons in the retrotrapezoid nucleus (RTN) provide a CO2/H+ -dependent drive to breathe and function as an integration center for the respiratory network, including serotonergic raphe neurons. We recently showed that serotonergic modulation of RTN chemoreceptors involved inhibition of KCNQ channels and activation of an unknown inward current. Hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels are the molecular correlate of the hyperpolarization-activated inward current (Ih) and have a high propensity for modulation by serotonin. To investigate whether HCN channels contribute to basal activity and serotonergic modulation of RTN chemoreceptors, we characterize resting activity and the effects of serotonin on RTN chemoreceptors in vitro and on respiratory activity of anesthetized rats. These results suggest that HCN channels contribute to resting chemoreceptor activity and that serotonin activates RTN chemoreceptors and breathing in part by a 5-HT7 receptor-dependent mechanism and downstream activation of Ih.

CENTRAL CHEMORECEPTION is the mechanism by which the brain regulates breathing in response to changes in CO2/H+. A region of the brainstem called the retrotrapezoid nucleus (RTN) is an important site of chemoreception. Neurons (Wang et al. 2013) and astrocytes (Gourine et al. 2010) in this region sense changes in CO2/H+ to produce an integrated CO2/H+ -dependent drive to breathe. Chemosensitive RTN neurons also integrate respiratory drive from other regions of the respiratory circuit such as serotonergic raphe neurons (Mulkey et al. 2007a). Serotonin is a potent modulator of breathing (Richerson 2004; Corcoran et al. 2014), including at the level of the RTN, where serotonin has been shown to stimulate chemoreceptor activity in vitro and increase breathing in conscious and anesthetized animals (Hawryluk et al. 2012). In addition, disruption of serotonergic signaling has been shown to decrease the respiratory chemoreflex (Hodges et al. 2008; Ray et al. 2011) and may contribute to respiratory failure associated with sudden unexpected death in epilepsy (SUDEP) (Massey et al. 2014; Buchanan et al. 2014). Despite this critical physiological role, the mechanisms by which serotonin activates RTN chemoreceptors have yet to be fully elucidated.

Serotonergic signaling is mediated through seven receptor families (5-HT1–7), including Gi (5-HT1)-, Gq (5HT2)-, and Gs (5HT4)-coupled receptors (Richer et al. 2003). In the RTN, serotonin sensitivity can be blocked with ketanserin (Mulkey et al. 2007a), an antagonist for both 5-HT2 and 5-HT3 receptors (Beique et al. 2004; Jasper et al. 1997). Evidence also indicates that serotonergic modulation of RTN neurons is determined, in part, by inhibition of KCNQ channels (Hawryluk et al. 2012), most likely by a 5-HT2 Gq-coupled receptor mechanism (Mulkey et al. 2007a,b; Delmas and Brown 2005). However, RTN chemoreceptors retain approximately half their serotonin sensitivity when KCNQ channels are blocked, suggesting involvement of other channels. Preliminary data suggest that when KCNQ channels are blocked, serotonin sensitivity of RTN neurons can be eliminated by inhibiting hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels (Hawryluk et al. 2012), which produce a hyperpolarization-activated inward current (Ih). HCN channels are known to be activated by cAMP (DiFrancesco and Totora 1991) and Gi-coupled receptor signaling (Ulens and Tytgat 2001). Therefore, we hypothesize that HCN channels contribute to serotonergic modulation of RTN chemoreceptors.

Here, we demonstrate the functional presence of both KCNQ and HCN channels in RTN chemoreceptors neurons. We show that inhibition of HCN channels increases ventilation in vivo and the firing rate of RTN chemoreceptors under control conditions but not when Ca2+-sensitive currents are blocked (Abbruzzese et al. 2010), suggesting that HCN channels dampen basal chemoreceptor excitability by a Ca2+-sensitive mechanism that may involve voltage-dependent Ca2+ channels, as described in other brain regions (Tsai et al. 2007). Inhibition of HCN channels also blunted serotonergic modulation of RTN neurons in vitro and attenuated the ventilatory response to serotonin in vivo. During
HCN and KCNQ blockade, serotonin failed to stimulate chemoreceptor activity or breathing, suggesting that coordinated activity of these channels is entirely responsible for serotonergic modulation of RTN chemoreceptors. Furthermore, inhibition of adenylate cyclase blocked the \( I_h \) component of the serotonergic response in RTN chemoreceptors, suggesting involvement of a G3-coupled receptor signaling. Consistent with this, serotonin-mediated activation of RTN chemoreceptors was blocked with a 5-HT-\( \gamma \)-receptor blocker and mimicked by a 5-HT-\( \gamma \)-receptor activator. We also find that serotonin shifted the voltage dependence of \( I_h \) activation to more depolarized potentials. These results build on our understanding of the mechanisms by which serotonin regulates breathing by identifying a novel role of HCN channels and 5-HT7 receptors in the serotonergic modulation of RTN chemoreceptors. Furthermore, considering that HCN and KCNQ are epilepsy-associated ion channels and disruption of serotoninergic signaling may contribute to SUDEP, these results suggest that HCN and KCNQ channels are common substrates for respiratory dysfunction and epilepsy.

**METHODS**

*Animals.* Animal use was in accordance with guidelines approved by the Universities of Connecticut and Sao Paulo Institutional Animal Care and Use Committees. The in vivo experiments were performed on male Wistar rats weighing 250–300 g (8–10 mo old). In vitro experiments were performed on brain slices isolated from rat pups (7–12 days postnatal).

*In vivo preparation.* The surgical procedures and experimental protocols were done as described previously (Hawryluk et al. 2012). Briefly, general anesthesia was induced with 5% halothane in 100% \( \text{O}_2 \). Artificial ventilation with 1.4–1.5% halothane in 100% \( \text{O}_2 \) was maintained throughout surgery. Standard surgical procedures (bilateral vagotomy, arterial cannulation, phrenic nerve dissection, and dorsal transerebellar access to the ventrolateral medulla oblongata) were used. After surgery, halothane was gradually replaced by urethane (1.2 g/kg, administered intravenous over 20 min). This initial anesthetic was stable for the duration of the experiment (up to 4 h after initial anesthetic crossover). Rats were ventilated with 100% \( \text{O}_2 \) supplied by artificial ventilation to increase the maximum end-expiratory \( \text{CO}_2 \) to 9.5–10%.

Serotonin was injected by pressure (40–60 psi, 4-ms pulses, 50 nl in 3–5 s) through glass pipettes (20-\( \mu \)m outside diameter) filled with 1 mm serotonin creatinine sulfate in a pH 7.3 normal saline solution containing 1% (vol/vol) fluorescent microbeads (for histological verification of injection sites). The concentration of serotonin used in these experiments was based on the EC50 response elicited by serotonin injections into the hypoglossal nucleus of anesthetized rats (Fenik and Veasey 2003). The pipette tip was placed 200 \( \mu \)m ventral to the caudal edge of the facial motor nucleus under electrophysiological guidance as described below for drug injections. In all cases, correct injection was verified by postmortem histological inspection of the location of fluorescent microbeads (Lumadour, New City, NY).

*Histology.* At the end of each in vivo experiment, rats were deeply anesthetized with halothane and perfused through the heart with PBS, pH 7.4, followed by paraformaldehyde (4% in 0.1 m phosphate buffer, pH 7.4). The brains were removed, and the medulla was cut into 40-\( \mu \)m-thick coronal sections (Vibratome 1000S Plus). We confirmed that injection sites were located within the RTN by fluorescent visualization using an Axioskop 2 microscope (Carl Zeiss, Thornwood, NY). Sections from different brains were aligned with respect to a reference section, which was the most caudal section containing an identifiable cluster of facial motor neurons. This reference section was assigned a value of 11.6 mm caudal to bregma (bregma: –11.6 mm; Paxinos and Watson 1989).

*Brain slice preparation and slice-patch electrophysiology.* As described (Mulkey et al. 2004; Wenker et al. 2012b), neonatal rats (P7–12 days postnatal) were decapitated under ketamine/xylazine anesthesia, and transverse brainstem slices (300 \( \mu \)m) were cut using a microslicer (Dsk 1500E; Dosaka) in ice-cold substituted Ringer solution containing (in mm): 130 NaCl, 3 KCl, 2 MgCl2, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, 10 glucose, and 1 kynurenic acid. Slices were incubated for ~30 min at 37°C and subsequently at room temperature in normal Ringer solution containing the following (in mm): 130 NaCl, 3 KCl, 2 MgCl2, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 glucose. All solutions were equilibrated with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \), extracellular pH 7.35.

Individual slices containing the RTN were transferred to a recording chamber mounted on a fixed-stage microscope (Zeiss Axioskop FS) and perfused continuously (~2 ml min\(^{-1}\)) with normal Ringer solution bubbled with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \). Hypercapnic solution was made by equilibrating normal Ringer solution with 15% \( \text{CO}_2 \) balance \( \text{O}_2 \) (pH~6.90). All recordings were made from neurons located within 100 \( \mu \)m of the ventral surface and below the caudal end of the facial motor nucleus using an Axopatch 200B patch-clamp amplifier, digitized with a Digitida 1322A A/D converter, and recorded using pCLAMP 10.0 software (Molecular Devices, Sunnyvale, CA). Recordings were obtained at room temperature (22°C) with electrodes \( \text{CO}_2 \) and PND appeared to have reached equilibrium. The CO2 PND threshold was determined as the percent CO2 that first elicited PND. End-expiratory CO2 was measured by averaging the maximum values recorded from 50 consecutive breaths at the midpoint of the time interval sampled. One arbitrary unit represents the highest value of PND measured at steady state with end-expiratory CO2 set at 9.5–10%.

Serotonin was injected by pressure (40–60 psi, 4-ms pulses, 50 nl in 3–5 s) through glass pipettes (20-\( \mu \)m outside diameter) filled with 1 mm serotonin creatinine sulfate in a pH 7.3 normal saline solution containing 1% (vol/vol) fluorescent microbeads (for histological verification of injection sites). The concentration of serotonin used in these experiments was based on the EC50 response elicited by serotonin injections into the hypoglossal nucleus of anesthetized rats (Fenik and Veasey 2003). The pipette tip was placed 200 \( \mu \)m ventral to the caudal edge of the facial motor nucleus under electrophysiological guidance as described below for drug injections. In all cases, correct injection was verified by postmortem histological inspection of the location of fluorescent microbeads (Lumadour, New City, NY).
Spike 5.0 software. Whole cell current-clamp recordings were made using internal solution containing the following (in mM): 125 potassium gluconate, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 4 Mg-ATP, 0.3 Na-GTP, 0.1 EGTA, 10 2-Tris-phosphocreatine, and 0.05% biocytin (pH 7.3). Cells were held at −60 mV, and depolarizing steps from −100 pA were applied (Δ +10 mV). Input resistance (\(R_{\text{in}}\)) was calculated from the voltage responses to hyperpolarizing steps. Initial spike frequency was measured following a 50-pA current injection, and the rebound spike latency was taken as the mean time to first action potential following hyperpolarizing steps (−100 to −70 pA). The sag ratio was expressed as \((1 - \Delta V_s/\Delta V_{\min}) \times 100\%\), where \(\Delta V_s = \text{resting membrane potential (RMP)} - V_t\) is the resting membrane potential, \(V_t\) is the steady-state potential, and \(V_{\min}\) is the initial minimum potential. Voltage-clamp recordings were made at a holding potential of −60 mV and in the presence of tetrodotoxin (TTX; 0.5 μM; Alomone Laboratories) to block action potentials and barium (1 mM) to block leak K\(^+\) channels. Holding current, conductance, and current-voltage (I–V) relationships were determined by applying hyperpolarizing voltage steps from −60 to −150 mV for 1,000 ms (Δ −10 mV). The maximal amplitude of \(I_h\) was quantified as the size of the time-dependent current during a step to −150 mV. The voltage dependence of \(I_h\) activation was obtained from tail currents measured at −70 mV following a series of hyperpolarizing steps; those data were normalized, plotted as a function of the initial step potential, and fitted to a Boltzmann function for calculation of voltage for half-maximal activation (\(V_{1/2}\)) of \(I_h\). For whole cell recordings series resistance (\(R_s\)) was typically <20 MΩ and was compensated by 65–70%. A liquid junction potential of 10 mV was corrected offline. Recordings were discarded if \(R_s\) varied >10% during an experiment.

Drugs. All drugs were purchased from Tocris Bioscience unless otherwise stated. For in vivo experiments, the KCNQ channel blocker XE991 (Abcam Biochemicals, Cambridge, MA) and the HCN channel blocker ZD7288 (Sigma-Aldrich) were diluted to 50 μM in sterile saline (pH 7.4) and injected into the RTN using a single-barrel glass pipette (tip diameter of 20 μm) connected to a pressure injector (Picospritzer III; Parker Hannifin, Cleveland, OH). Since the RTN is located bilaterally and both structures contribute to the chemoreflex, we tested for contributions of KCNQ and HCN channels to the CO\(_2\)/H\(_2\) drive to breathe we injected XE991 or ZD7288 bilaterally. However, since unilateral RTN injection of serotonin can elicite a ventilatory response, we also tested contributions of KCNQ and HCN to this response by making same side injections of the blockers. For each injection we delivered a volume of 50 nl over a period of 5 s. These glass pipettes also allowed for recordings of field-potential properties that were used to help direct the electrode tip to the desired site. Injections in the RTN region were guided by recordings of the facial field potential (Brown and Guyenet 1985) and were placed 250 μm below the lower edge of the field, 1.7 mm lateral to the midline, and 200 μm rostral to the caudal end of the field. Recordings were made on one side only; the second injection was made 1–2 min later at the same level on the contralateral side. We included a 2% dilution of fluorescent latex microbeads with all drug applications to mark the injection sites and verify the spread of the injections (Takaura and Moreira 2011; Takakura et al. 2011). For in vitro experiments, we bath-applied serotonin hydrochloride (5 μM; Sigma-Aldrich), XE991 (10 μM; Abcam Biochemicals) to block KCNQ channels, ZD7288 (50 μM), or CsCl (2 mM; Sigma-Aldrich) to block HCN channels, CdCl\(_2\) (100 μM; Sigma-Aldrich) to block voltage-gated calcium channels, synaptic block perfusate contained kynurenic acid (1 mM; Sigma-Aldrich) to block ionotropic glutamate receptors, bicuculline (10 μM; Sigma-Aldrich) to block GABA\(_A\) receptors and strychnine (20 μM; Sigma-Aldrich) to block glycine receptors, SQ22536 (100 μM) to inhibit adenylate cyclase activity, SB258719 (10 μM) to block 5-HT\(_7\) receptors, 5-carboxamidotryptamine (5-Ct, 5 μM) to activate 5-HT\(_7\), and WAY100635 (0.1 μM) to block 5-HT\(_1\) receptors and the specific 5-HT\(_3\) agonist LP-44 (2 μM).

Statistical analysis. Data are reported as means ± SE and illustrated as bar graphs with individual data points. Statistical analysis was performed using GraphPad Prism version 3.0 software. Data were assessed for normality, and the Student’s t-test or one-way ANOVA followed by Newman-Keuls multiple-comparisons test were used as appropriate. The relevant values used for statistical analysis are provided in RESULTS.

RESULTS

This study consists of three sets of experiments designed to address the mechanistic underpinnings of serotonin-induced respiratory activity in the RTN. First, to determine if serotonin-mediated cardiorespiratory control within the RTN involves HCN channels, we examined breathing and blood pressure in anesthetized rats injected with serotonin into the RTN, either alone or after injection of KCNQ and/or HCN channel blockers. KCNQ channels were blocked in a number of experiments to reveal the KCNQ-independent component to the serotonin response. Second, to determine whether HCN channels regulate excitability and serotonergic modulation of RTN chemoreceptors we tested effects of blocking HCN channels, alone and during blockade of KCNQ channels, on RTN neuron activity (basal firing rate and serotonin-sensitivity) in the brain slice preparation. Third, to identify mechanism(s) underlying serotonergic modulation of HCN channels in RTN chemoreceptors, we assessed serotonin responses when adenylate cyclase activity is blocked and tested the effects of subtype-specific serotonin receptor modulators on RTN chemoreceptor activity. Furthermore, in voltage-clamp experiments, we determined the effects of serotonin on amplitude and voltage-dependent activation of \(I_h\).

Here we focus on chemosensitive RTN neurons, which have been shown to express the transcription factor Phox2b (Lazarenko et al. 2009; Stornetta et al. 2006). However, Phox2b is also expressed by other cells in relatively close proximity to the RTN, including catecholaminergic neurons (C1 and A5), facial motor neurons, and the superior salivary nucleus (Kang et al. 2007). Therefore, we chose to functionally identify CO\(_2\)/H\(_2\) sensitive RTN neurons based on their characteristic firing rate response to CO\(_2\)/H\(_2\). Neurons were considered chemosensitive if they increased the firing rate by ≥1.5 Hz following exposure to 15% CO\(_2\). This level of CO\(_2\)/H\(_2\) response is similar to what we, and others, have reported for chemosensitive RTN neurons in vitro (Mulkey et al. 2006; Rittucci et al. 2005; Wenker et al. 2010). RTN neurons that did not exhibit this minimum response were considered nonchemosensitive and excluded from this study.

HCN channels in the RTN contribute to basal activity and serotonergic modulation of respiratory drive. Previous evidence indicates that KCNQ channels regulate activity and serotonergic modulation of RTN neurons (Hawryluk et al. 2012). Preliminary in vitro evidence also suggested that HCN channels are expressed in RTN neurons and contribute to serotonin responses (Hawryluk et al. 2012). To establish the functional significance of HCN channels in controlling breathing, we tested whether RTN injection of an HCN channel blocker (ZD7288) affects resting respiratory motor output of anesthetized rats. We then tested whether the ventilatory response to serotonin was altered by RTN injection of ZD7288. All injections (uni- or bilateral) were placed 250 μm below the
facial motor nucleus and 200 \mu m rostral to the caudal end of this nucleus to target the region containing the highest density of CO$_2$-sensitive RTN neurons (Mulkey et al. 2004; Takakura and Moreira 2011; Takakura et al. 2011) (Figs. 1E and 2B).

In anesthetized animals, we found that bilateral RTN injections of ZD7288 (50 \mu M, 50 nl each side) increased resting respiratory output as measured by a change in PND. As described previously, bilateral injections of XE991 into the RTN increased the neural equivalent of minute ventilation (mvPND, product of PND amplitude and frequency; Fig. 1, B and E). Bilateral injections of ZD7288 to inhibit HCN channels also increased respiratory activity by 48 \pm 7\% \left[F_{(3,50)} = 144.12, P < 0.01; \text{Fig. 1}, \text{A and B}\right]. Bilateral blockade of both KCNQ and HCN within the RTN did not increase respiratory activity further than injection of either blocker alone (Fig. 1, A and B). We also found that the ventilatory response to a maximum stimulus of 10\% CO$_2$ was unaffected by application of XE991 and/or ZD7288 into the RTN [Fig. 1, A and C; $F_{(3,50)} = 0.0254, P > 0.05$]. However, bilateral application of XE991 and/or ZD7288 shifted the CO$_2$ threshold (i.e., the level of CO$_2$ required to stimulate ventilatory output) from 5.3 \pm 0.09 to 4.5 \pm 0.1\% \left[F_{(3,50)} = 87.44, P < 0.01; \text{Fig. 1D}\right]. These data suggest that inhibition of KCNQ and HCN channels increases excitability of RTN chemoreceptors and consequently the respiratory system responses to changes in CO$_2$.

In agreement with previous studies (Hawryluk et al. 2012; Mulkey et al. 2007a), unilateral RTN injection of serotonin (1 mM) increased PND amplitude by 37 \pm 5\% above baseline \left[F_{(3,29)} = 129.83, P < 0.01; \text{Fig. 2, A and C}\right]. After respiratory activity returned to control levels, unilateral RTN injection of XE991 (50 \mu M, 50 nl) or the HCN blocker ZD7288 (50 \mu M, 50 nl) had no discernible effect on resting PND \left[F_{(3,29)} = 0.011, P > 0.05; \text{Fig. 2A}\right]. However, injection with either channel blocker significantly blunted, and together essentially eliminated, serotonin modulation of respiratory activity (Fig. 2, A and C). For example, injection of serotonin after application of either XE991 (Fig. 2A1) or ZD7288 (Fig. 2A2) only increased PND amplitude 22 \pm 2 and 29 \pm 3\% above baseline, respectively. This reflects a 40 \pm 3 and 21 \pm 2\% decrease in serotonin responsiveness, respectively, relative to control conditions \left[F_{(3,29)} = 167.74, P < 0.01; \text{Fig. 2, A and C}\right]. After coapplication of both XE991 and ZD7288 into the RTN, subsequent injection of serotonin only increased PND amplitude by 5 \pm 2\% (Fig. 2C). Serotonin injection into this region also decreased MAP by 15 \pm 4 mmHg, and this hypotensive response was unaffected by XE991 or ZD7288 \left[F_{(3,29)} = 0.0012, P > 0.05; \text{Fig. 2A}; \text{summary data not shown}\right], suggesting that KCNQ and HCN channels are not involved in cardiovascular regulation at this level of the rostral ventrolateral medulla. These results demonstrate that KCNQ channels and HCN channels within the RTN serve as functional determinants of serotonergic modulation of respiratory drive.

HCN channels contribute to both resting firing behavior and serotonergic modulation of RTN chemoreceptors in vitro. To examine the contribution of HCN channels to activity of RTN chemoreceptors, we first characterized membrane potential responses to hyperpolarizing and depolarizing current steps under control conditions and during HCN channel blockade. In the whole cell current-clamp configuration, chemosensitive RTN neurons have an $R_{m}$ of 910 \pm 83 M\Omega and exhibit a sag ratio of 32.3 \pm 4.6\% in response to a -100-pA hyperpolarizing voltage step (Fig. 3A). At the end of the hyperpolarizing step, chemosensitive RTN neurons produce a rebound spike with a latency of 109 \pm 19 ms (Fig. 3A4). Bath application of ZD7288 (50 \mu M) increased $R_{m}$ to 1196.0 \pm 64.5 M\Omega, eliminated the sag (to a sag ratio of only 3.1 \pm 4.5\%); $T_{(5)} = 5.96, P = 0.0019; \text{Fig. 3A2}, and increased the rebound spike latency $T_{(5)} = 3.68, P = 0.0142; \text{Fig. 3A4}$. We also found that the firing rate response to a depolarizing current injection (20 pA) was unaffected by ZD7288 (Fig. 3A3). This is not surprising considering that HCN channels are inhibited by depolarization. Furthermore, blocking KCNQ channels with XE991 (10 \mu M) caused a modest increase in $R_{m}$ $T_{(4)} = 4.71, P = 0.0093$) but with no effect on the sag potential or rebound spike latency

Fig. 1. Hyperpolarization-activated cyclic-nucleotide-gated (HCN) channels in the retrotrapezoid nucleus (RTN) regulate resting breathing activity and the ventilatory response to CO$_2$ in anesthetized rats. A: traces of end expiratory CO$_2$ (etCO$_2$), arterial pressure (AP), and integrated phrenic nerve discharge (iPND) show the respiratory response to bilateral injections (arrows) of saline or HCN channel inhibitor ZD7288 in the RTN. Under control conditions, injections of ZD7288 (50 \mu M, 50 nl each side) increased respiratory activity with an increase in mvPND (product of PND amplitude and frequency) and significantly lowered PND CO$_2$ threshold (D). However, CO$_2$ responsiveness was otherwise unaffected by application of ZD7288 in the RTN; lowering etCO$_2$ from 3 to 4\% inhibited respiratory output, and graded increases etCO$_2$ up to 9–10\% increased mvPND by an amount similar to saline (control). B: summary data plotted as change in mvPND shows the effect of ZD7288 alone (50 \mu M) or in combination with KCNQ channel inhibitor XE991 (50 \mu M) on resting respiratory activity ($n$ = 7 animals). C: summary data showing CO$_2$-induced changes in mvPND under control conditions, after injections of ZD7288 or XE991 or after injections of ZD7288 + XE991 ($n$ = 7 animals). D: summary data showing that ZD7288 alone or in combination with XE991 decreased the level of CO$_2$ required to stimulate PND activity ($n$ = 7 animals). E: computer-assisted plots of the center of the injection sites (coronal projection on the plane Bregma: –11.6 and –11.3 mm; Paxinos and Watson 1989). Note that all injections were made in the caudal aspect of the RTN where there is the highest density of chemosensitive RTN neurons. Sp5, spinal trigeminal tract; py, pyramids; VII, facial motor nucleus; au, arbitrary units. \*P < 0.01.

\[I_n \text{ CONTRIBUTES TO SEROTONERGIC MODULATION OF BREATHING}\]
ability of RTN chemoreceptors, we tested the effects of HCN and KCNQ channels on chemoreceptor activity [Fig. 3B]. In addition, XE991 also increased the instantaneous spike frequency elicited by 20-pA current injections (Fig. 3B4). These results indicate that HCN and KCNQ channels regulate intrinsic electrical properties of chemosensitive RTN neurons.

Next, to determine whether HCN channels regulate excitability of RTN chemoreceptors, we tested the effects of HCN channel blockers (ZD7288 or Cs+) on basal activity in the rat brainstem slice preparation. We also used HCN channel blockers alone and in conjunction with XE991 to test for contributions of HCN channels to the serotonin response of RTN chemoreceptors. We found that under control conditions inhibition of HCN channels with ZD7288 (50 μM) or Cs+ (2 mM) increased chemoreceptor activity by 1.01 ± 0.17 Hz \(T_{\text{16}} = 5.93, P < 0.0001\); Fig. 4, A and B], suggesting that HCN channels can limit basal activity of RTN chemoreceptors. Note that there was no difference in the effects of ZD7288 and Cs+ on chemoreceptor activity \(T_{\text{15}} = 1.497, P = 0.1551\) so data were pooled. Based on evidence from other brain regions, HCN channels may inhibit neural activity by shunting synaptic potentials (Stuart and Spruston 1998) or by influencing activity of other voltage-dependent channels including KCNQ (George et al. 2009) and Ca2+ channels (Tsay et al. 2007). To determine which of these possibilities may contribute to the inhibitory effects of HCN channel on resting activity of RTN chemoreceptors, we characterized effects of HCN channel blockade 1) when excitatory and inhibitory receptors are blocked with a cocktail containing kynurenic acid (1 mM), bicuculline (10 μM), and strychnine (20 μM) (i.e., synaptic blockade); 2) when KCNQ channels were blocked with XE991; and 3) when voltage-gated Ca2+ channels were blocked with Cd2+ (100 μM). We found that the firing rate response of RTN chemoreceptors to HCN channel blockade (i.e., bath application of ZD7288 or Cs+) was unaffected by synaptic blockade but was reduced to a change of 0.38 ± 0.16 Hz in XE991 and eliminated (0.08 ± 0.06 Hz) in Cd2+ \(F_{\text{13,39}} = 7.13, P = 0.0006\); Fig. 4, A and B], suggesting that HCN channels limit basal activity of RTN chemoreceptors indirectly by mechanisms involving KCNQ and Cd2+-sensitive channels including Ca2+ channels.

As expected, bath application of serotonin (5 μM) increased activity of these cells by 1.18 ± 0.13 Hz [paired \(T_{\text{14}} = 9.00, P < 0.001\); Fig. 4, A and C] and it did so in a...
reversible and repeatable manner (ratio of the third serotonin response divided by the second response was 0.9 ± 0.1; Hawryluk et al. 2012). Bath application of XE991 (10 μM) to block KCNQ channels increased baseline firing by 1.04 ± 0.43 Hz [paired T(10) = 5.42, P = 0.0003] and blunted serotonin responsivity by 54.3 ± 11.9% [F(3,38) = 20.06, P < 0.0001; Fig. 4, A and C], as previously reported (Hawryluk et al. 2012). Application of ZD7288 (50 μM) to block HCN channels (with DC current injection to adjust baseline to control levels) reduced subsequent serotonin response to an increase of only 0.57 ± 0.09 Hz (Fig. 4, A and C). Notably, when both KCNQ and HCN channels are blocked with XE991 and ZD7288 or Cs+ (with baseline activity adjusted by DC current injection to near control levels), serotonin increased firing rate less than under control conditions (Fig. 4, A and C), i.e., serotonin increased activity by only 0.11 ± 0.04 Hz in XE991 plus ZD7288 and only 0.07 ± 0.17 Hz in XE991 plus Cs+. When the inhibitory contribution of HCN channel activity was blocked by Cd2+, exposure to serotonin stimulated chemoreceptor activity by an amount similar to that of control conditions [T(5) = 0.66; P = 0.5407; Fig. 4, A2 and F1], as expected for activation of an inward depolarizing current. It should be noted that CO2/H+ sensitivity of RTN neurons was retained when HCN channels were blocked with ZD7288 or Cs+ (Fig. 4, D and E). These results suggest that both KCNQ and HCN channels are essential components of serotonergic modulation in RTN chemoreceptor neurons. Collectively, these results also suggest that the net contribution of HCN channels to RTN chemoreceptor basal activity is to limit excitability indirectly by influencing activity of KCNQ and Ca2+ channels. However, targeted activation of presumably a select subset of HCN channels by serotonin can increase chemoreceptor activity and contribute to the excitatory effects of serotonin on breathing.

Serotonin activates Ih in RTN neurons by a Gi-coupled receptor and cAMP-dependent mechanism. It is unlikely that a single serotonin receptor subtype mediates these divergent effects on KCNQ and HCN channels. HCN channels are known to be activated by Gi-coupled receptors (Ulens and Tygat 2001) but inhibited by Gi-coupled receptor signaling (Lui et al. 2003), indicating that HCN channel activity may be regulated by diverse signal transduction pathways. Gi-coupled receptor signaling involves activation of adenylyl cyclase and increased cAMP production (Wetschereck and Offermanns 2005), and it is well known that cAMP can directly bind HCN.
Channels to facilitate $I_h$ (DiFrancesco et al. 1991). We therefore tested the possibility that serotonin activates HCN channels by $G_i$-coupled receptor signaling.

We first sought to determine if adenylate cyclase activity is required for serotonin-mediated activation of $I_h$. To study serotonin modulation of HCN channels in relative isolation, we performed these experiments in the presence of XE991 (10 μM) to block KCNQ channels. As before, KCNQ channel blockade decreased the firing rate response to serotonin [control, $1.34 \pm 0.21$ Hz; XE991, $0.81 \pm 0.22$ Hz; $F_{(1,4)} = 11.38$, $P = 0.0046$; Fig. 5, A and C]. During KCNQ channel blockade (and after baseline activity was adjusted by DC current injection to near control levels), application of the adenylate cyclase inhibitor SQ22536 (100 μM) increased the firing rate of RTN chemoreceptors by $0.80 \pm 0.70$ Hz [$T_{(6,21)} = 6.43$, $P = 0.0030$; Fig. 5, A and B]. However, when HCN channels are blocked with Cs$^+$ (2 mM), bath application of SQ22536 still increases in the chemoreceptor activity by an amount similar to that under control conditions ($n = 3$; data not shown), suggesting cAMP can modulate activity of RTN chemoreceptors by mechanisms in addition to activation of HCN. Furthermore, in the presence of SQ22536 inhibition of HCN with Cs$^+$ increased RTN chemoreceptor activity by an amount similar to that under control conditions ($n = 4$; data not shown), suggesting that cAMP is not required for the maintenance of HCN channel activity under basal conditions. This latter finding is consistent with the possibility that the inhibitory contribution of HCN to basal activity of RTN neurons is determined indirectly by the voltage-dependent modulation of KCNQ and Ca$^{2+}$ channels. Nevertheless, in the presence of XE991 and SQ22536, exposure to serotonin increased the firing rate by only $0.25 \pm 0.06$ Hz [$F_{(2,4)} = 11.38$, $P = 0.0046$; Fig. 5, A and C]. These findings suggest that adenylate cyclase activity is required for the $I_h$ component of serotoninergic modulation of chemosensitive RTN neurons.

In other brain regions, cAMP-mediated activation of HCN channels results from a depolarizing shift in voltage-dependent activation of $I_h$ (DiFrancesco and Totora 1991). To confirm this is the case for RTN chemoreceptors, we characterized effects of serotonin on the maximum amplitude and voltage-dependent activation of $I_h$. Maximum $I_h$ amplitude was defined as the difference between instantaneous and steady-state currents during a $-150$-mV step (represented in Fig. 6A, by dashed lines). In the whole cell voltage-clamp configuration (holding potential of $-60$ mV and in TTX [0.5 μM] to block action potentials), $I_h$ was detected by its characteristic time-dependent activation during hyperpolarizing voltage steps. The maximum amplitude of $I_h$ in RTN chemoreceptors was $64.55 \pm 14.53$ pA under control conditions and $57.1 \pm 12.5$ pA during exposure to serotonin (Fig. 6B). In addition, bath application of ZD7288 largely eliminated $I_h$ [$F_{(2,21)} = 5.21$, $P = 0.0146$; Fig. 6B]. These results suggest that serotonin does not affect maximum $I_h$ in these cells.

To determine the effects of serotonin on the voltage dependency of $I_h$ activation, we measured tail currents at a fixed
potential of \(-70\) mV under control conditions and after activating \(I_h\) with a series of hyperpolarizing voltage steps. Normalized \(I_h\) tail currents were plotted as a function of membrane potential during the initial hyperpolarizing steps and fitted with a Boltzmann function to determine the half-activation voltage (\(V_{1/2}\)) (Sirois et al. 2002; Wenker et al. 2012a). The \(V_{1/2}\) for \(I_h\) activation averaged \(-120.1 \pm 3.2\) mV in control conditions and \(-103.7 \pm 6.0\) mV in serotonin [\(T_{(4)} = 2.35, P = 0.0394\); Fig. 6C], indicating that serotonin caused a depolarizing shift of \(-16\) mV in the voltage-dependent activation of \(I_h\). These results demonstrate \(I_h\) within RTN chemoreceptor neurons and strongly suggest that serotonin activates HCN channels in the RTN by a G, - and cAMP-dependent mechanism.

Several members of the serotonin receptor family are G-coupled including 5-HT_4, 5-HT_6, and 5-HT_7. Of these, 5-HT_7 receptors in particular are inhibited by ketanserin (Lesage et al. 1998; Shen et al. 1993). Since ketanserin was shown to block the serotonin responses of chemosensitive RTN neurons (Mulkey et al. 2007a), we considered the possibility that 5-HT_7 receptors mediate the effects of serotonin on \(I_h\). To test for involvement of 5-HT_7 receptors, we used 5-CT, which activates 5-HT_7 as well as several G-coupled receptors (i.e., 5-HT_1A/1B/1D/2B), and WAY100635, which selectively blocks 5-HT_1A receptors (Beique et al. 2004). If exposure to 5-CT mimicked effects of serotonin on chemoreceptor activity under control and WAY100635-exposed conditions by a \(I_h\)-dependent mechanism, this would suggest that 5-HT_7 receptors contribute to serotonin-mediated activation of HCN channels in RTN neurons. Finally, we tested this by using the 5-HT_7-specific antagonist SB258719, along with the 5-HT_7 receptor agonist LP-44.

We found that bath application of 5-CT (5 \(\mu M\)) increased chemoreceptor activity from 0.97 \pm 0.37 to 1.48 \pm 0.41 Hz [\(T_{(12)} = 7.02, P < 0.0001\); Fig. 7, A and B]. This change in activity (0.52 \pm 0.07 Hz) was similar in magnitude to the serotonin response when KCNQ channels were blocked (Fig. 4C). A second exposure to 5-CT, this time when HCN channels were blocked with ZD7288, failed to significantly increase the firing rate [with a rise of only 0.16 \pm 0.05 Hz; \(F_{(4,27)} = 7.83, P = 0.0003\); Fig. 7, A and B]. Bath application of WAY100635 had no effect on baseline activity [\(T_{(6)} = 1.67, P = 0.1452\)] or firing-rate response to 5-CT [\(F_{(4,27)} = 7.83, P = 0.0003\); Fig. 7B], suggesting that 5-HT_1A receptors do not exert direct or indirect control (e.g., modulation of inhibitory input) over chemosensitive RTN neurons in vitro. Application of a 5-HT_7 receptor blocker (SB258719, 10 \(\mu M\)) reduced the firing rate response of RTN chemoreceptors to serotonin by approximately half [\(T_{(3)} = 6.98, P = 0.006\); data not illustrated] and eliminated the 5-CT response [\(T_{(4)} = 3.70, P = 0.0208\); Fig. 7C]. Furthermore, the 5-HT_7 agonist LP-44 (2 \(\mu M\)) induced a 0.77 \pm 0.16 Hz increase in the chemoreceptor firing rate and this response was blocked by a 5-HT_7 antagonist [SB258719, 10 \(\mu M\]; \(T_{(3)} = 5.56, P = 0.0309\); Fig. 7D]. Together, these results suggest that 5-HT_7 receptors mediate the effects of serotonin on HCN channels in RTN neurons.

**DISCUSSION**

The RTN is an important locus of respiratory control, as neurons in this region provide a CO_2/H^+-dependent drive to breathe (Mulkey et al. 2004; Wang et al. 2013) and serve as a point of convergence for other respiratory centers including the medullary raphe (Hawryluk et al. 2012; Mulkey et al. 2007a). Despite the importance of RTN chemoreceptors in breathing, we are only just beginning to understand the ionic mechanisms that control their resting membrane potential and response to neurotransmitters. Recent evidence indicates that KCNQ channels in RTN neurons are key determinants of resting firing behavior and serotoninergic modulation of breathing (Hawryluk et al. 2012). Here we show that HCN channels are expressed in RTN chemoreceptor neurons and are a second essential regulator of serotonin-mediated chemoreceptor function. Specifi-
nize that the level of CO$_2$ used here to identify chemosensitive channels, e.g., well as by modulating activity of other voltage-dependent inhibits neuronal activity by decreasing input resistance, as unlikely to produce common off target effects. It should old potentials that can have opposing influences on neuronal increase neuronal excitability by depolarizing membrane po-

et al. 2010). Therefore, additional experiments are required to increase by warming from room to body temperature. Therefore, it is possible that we underestimate the extent to which these channels contribute to chemoreceptor activity. However, we have shown previously that chemosensitive RTN neurons in slices from rat pups (recorded at room temperature) respond to raphe neurotransmitters (e.g., serotonin) in a manner similar to chemosensitive RTN neurons in anesthetized adult rats (Mulkey et al. 2007a). Therefore, it is unlikely that the fundamental mechanisms underlying neurotransmitter modulation of these neurons (i.e., HCN channel modulation) differ significantly with age or temperature. Although ZD7822 is a commonly used HCN channel blocker, there is some evidence that it can also block Na$^+$ and Ca$^{2+}$ channels (Wu et al. 2012). Therefore, to minimize potential off target effects, we also used a second HCN channel block (Cs$^+$) that is chemically distinct and unlikely to produce common off target effects. It should also be noted that Cd$^{2+}$, in addition to blocking Ca$^{2+}$ channels, can also block certain voltage-gated K$^+$ channels (Abbruzzese et al. 2010). Therefore, additional experiments are required to confirm involvement of Ca$^{2+}$ channels. Lastly, we also recognize that the level of CO$_2$ used here to identify chemosensitive RTN neurons is high. However, we consider this a minor issue because cells identified in vitro as chemoreceptors using 15% CO$_2$ appear to be of the same population of cells identified in vivo as RTN chemoreceptors, i.e., located in the same region, are glutamatergic and express Phox2B (Mulkey et al. 2004; Mulkey et al. 2007b).

HCN channels generate an inward current ($I_h$) at subthresh-
old potentials that can have opposing influences on neuronal excitability. For example, activation of $I_h$ has been shown to increase neuronal excitability by depolarizing membrane potential (Wenker et al. 2012a). However, activation of $I_h$ also inhibits neuronal activity by decreasing input resistance, as well as by modulating activity of other voltage-dependent channels, e.g., $I_h$-mediated depolarization can increase activity of KCNQ channels (George et al. 2009) and prolong inactivation of Ca$^{2+}$ channels (Tsay et al. 2007). Therefore, both activation and inhibition of HCN channels may cause similar effects on neural activity. Furthermore, the net contribution of $I_h$ to RTN chemoreceptor activity likely depends on several factors including subcellular HCN channel distribution and proximity to other voltage-gated ion channels and/or neu-

rotransmitter receptors. Here, we show in vitro that blocking HCN channels increased the firing rate of RTN neurons under control conditions. Likewise, bilateral RTN injections of ZD7288 increased basal respiratory activity in anesthetized rats. We also found in vitro that the firing rate response of RTN chemoreceptors to HCN channel blockade was blunted by XE991 and eliminated by Cd$^{2+}$, suggesting that under resting conditions HCN channels limit chemoreceptor activity by mechanisms involving KCNQ and Ca$^{2+}$-sensitive channels including Ca$^{2+}$ channels. Consistent with a contribution of KCNQ channels to the inhibitory effects of HCN channel blockade, we found in vivo that bilateral RTN injections of ZD7288 and XE991 increased respiratory output by an amount similar to either drug alone (i.e., the responses were not additive). These results are consistent with evidence from other brain regions that showed HCN-mediated depolarization can enhance activity of voltage-dependent KCNQ channels and in doing so produced a net inhibitory influence on neuronal excitation (George et al. 2009). An additional inhibitory effect of HCN channel activation has been shown to result from prolonging Ca$^{2+}$ channel inactivation (Tsay et al. 2007). Importantly, the excitatory effect of serotonin on chemoreceptor activity was retained in Cd$^{2+}$, as expected for activation of an inward current. These results suggest that the excitatory and inhibitory effects of HCN channel activity can coexist in the same cells and can contribute to RTN chemoreceptor activity by different mechanisms.

Serotonergic modulation of RTN chemoreceptor neurons appears to be intrinsic. For example, previous (Mulkey et al. 2007a) and present results suggest that in the presence of TTX (to block action potentials) RTN chemoreceptors respond to serotonin with a modest decrease in holding current. Further-

Fig. 8. Model depicting the molecular basis for serotonergic modulation of RTN chemoreceptors. Previous (Hawryluk et al. 2012) and present results show that serotonin stimulates activity of RTN chemoreceptors by 2 independent but coordinated signaling pathways: 5-HT$_1$-mediated inhibition of KCNQ channels most likely by $G_q$ signaling, and 5-HT$_2$-mediated activation of HCN channels by a mechanism involving activation of adenylate cyclase- and cAMP-mediated depolarizing shift in the voltage-dependent activation of $I_h$. Our evidence suggests that each of these signaling pathways can independently stimulate activity of RTN chemoreceptors and together their coordinated activity ensures a robust response to serotonin. Note that our evidence also suggests that under basal conditions HCN channel activity may indirectly modulate activity of voltage-dependent KCNQ and Cd$^{2+}$-sensitive channels including Ca$^{2+}$ channels.
more, we show here that RTN chemoreceptors exhibit a characteristic $I_h$ that was activated by serotonin, suggesting a direct effect of serotonin on HCN channels in RTN chemoreceptors. Although RTN astrocytes are also known to contribute to the mechanism of chemoreception (Gourine et al. 2010; Huckstepp et al. 2010; Wenker et al. 2012), presumably by a TTX-independent mechanism, preliminary results suggest that CO$_2$/H$^+$-sensitive RTN astrocytes do not exhibit a serotonin-sensitive current (data not shown). However, there is some evidence that astrocytes in other brain regions express 5-HT receptors (Hirst et al. 1997; Miyazaki et al. 2013) and so it remains possible that astrocytes influence serotonergic modulation of chemoreception at other levels of the respiratory network.

The role we are attributing to HCN channels in chemosensitive RTN neurons differs from that previously described for CO$_2$-inhibited medullary neurons isolated from fetal rats, where inhibition of HCN channels decreased resting activity and blocked CO$_2$/H$^+$ sensitivity (Wellner-Kienitz and Shams 1998). The divergent roles of $I_h$ in these cell types may be due to developmental changes in the expression of HCN channel subunits as described in other brainstem regions (Bayliss et al. 1994); however, this possibility requires further investigation.

Our working model for serotonergic modulation of RTN chemoreceptor activity is summarized in Fig. 8. Ketanserin has been shown to block serotonin-stimulated activity of RTN neurons (Mulkey et al. 2007a), suggesting involvement of 5-HT$_2$- and/or 5-HT$_1$-receptors. It is well known that 5-HT$_2$-receptors are coupled to the phosphoinositol (i.e., $G_q$-coupled) second messenger system, which can modulate ion channel function by depleting plasma membrane levels of phosphatidylinositol 4,5-bisphosphate (PIP2) and by formation of inositol 1,4,5-trisphosphate and diacylglycerol (Suh and Hille 2002). Although not tested directly in this study, it is likely that activation of 5-HT$_2$-receptors and subsequent PIP2 depletion are the likely mechanisms by which serotonin inhibits KCNQ channels in RTN neurons (Hawryluk et al. 2012; Delmas and Brown 2005). It is also well established that 5-HT$_1$-receptors are coupled to trimeric $G$-proteins and when activated stimulate adenylate cyclase production of cAMP followed by activation of protein kinase A (PKA) (Matthys et al. 2011). We show that activation of HCN channels via this signaling pathway also contributes to serotonergic modulation of RTN chemoreceptors. Although activation of each of these pathways can independently increase neuronal activity, simultaneous activation of both may antagonize the downstream effects on KCNQ and HCN channels. For example, $G_q$-mediated PIP2 depletion (Pian et al. 2006) and PKC activation (Lui et al. 2003) have been shown to inhibit $I_h$ whereas $G_i$-mediated activation of PKA may activate certain KCNQ channels (Schröder et al. 1998). It is possible that coordinated activation of both $G_q$ and $G_i$ cascades ensures a robust serotonin response, while cross talk between pathways prevents overactivation.

In summary, our data identify HCN channels as key determinants of both excitability and serotonergic modulation of RTN chemoreceptor neurons and breathing. The mechanism by which serotonin activates HCN channels in RTN chemoreceptor neurons involves $G_q$-coupled receptor signaling via 5-HT$_1$-receptors.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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