Characterization of the chemosensitive response of individual solitary complex neurons from adult rats

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PUTATIVE CENTRAL CHEMOSENSITIVE neurons have mostly been studied in neonatal rats in various localized regions within the brain stem, including the solitary complex (SC), the medullary raphe, the nucleus of the solitary tract, intracellular pH, membrane potential; ventilation; chemosensitivity; gap junctions

Methods

Solutions. Artificial cerebral spinal fluid (aCSF) contained the following (in mM): 124 NaCl, 5.0 KCl, 2.4 CaCl2, 1.24 MgSO4, 1.24 NaHCO3, 1.25 NaH2PO4, 25 glucose, and 0.14 MgSO4. The pH of the aCSF was adjusted to 7.40 by adding NaHCO3. The aCSF contained 10 mg/l of insulin, 100 mg/l of penicillin, 100 mg/l of streptomycin, and 1% bovine serum albumin (http://www.ajpregu.org).
K₃HPO₄, 26 NaHCO₃, and 10 glucose, and was equilibrated with 95% O₂/5% CO₂ [extracellular pH (pHₑ) = 7.45 at 37°C]. The HA solution was identical to aCSF but was equilibrated with 85% O₂/15% CO₂ (pHₑ = 6.8–6.9 at 37°C). Synthetic blockade (SNB) solution was modified from aCSF and contained 0.2 mM CaCl₂ and 11.4 mM MgSO₄ ([NaCl] was adjusted to maintain osmotic balance) (5, 7). Carbenoxolone (CARB; 100 μM) was added to SNB solution to block electrical transmission (5, 9). Isohydric hypercapnic (ICP) solution was modified from aCSF with NaHCO₃ increased to 77 mM and NaCl decreased to 73 mM to keep pHₑ and osmotic pressure the same. The whole cell patch intracellular solution contained (in mM): 130 K⁺-glucuronate, 10 K⁺-HEPES, 0.4 EGTA, 1 MgCl₂, 0.3 GTP, and 2 ATP (pH = 7.45 at room temperature). This solution was used to reduce potential washout of the chemosensitive response (5, 15). The high K⁺/nigericin solution that was used for calibration (50) in the imaging studies contained (in mM): 104 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.24 KH₂PO₄, 25 N-methyl-D-glucamine (NMDG)-HEPES, 25 K-HEPES, 10 glucose, and 0.004 nigericin titrated with KOH or HCl to a pH value ranging from 6.2–8.6. The NH₄Cl solution was modified from aCSF with 104 mM NaCl to adjust for the added 20 mM NH₄Cl. 0.2003)/(2.0194 

Measurement of intracellular pH. pHₑ was measured as previously described (44). Individual SC neurons were loaded with 1 mM of the pH-sensitive dye, pyranine, through a whole cell patch pipette. The pipette was filled with whole cell patch solution (see above). Briefly, a whole cell patch pipette (5 MΩ) was fabricated from borosilicate glass using a Narishige PP-830 dual-stage pipette puller. The pipette was filled with whole cell patch solution (see above). Positive pressure was maintained on the pipette as a −0.1-nA pulse (30 ms, 5 Hz) was applied. Tip impedance increased as the pipette approached a neuron, which was indicated by a 1–2-mV downward deflection. Negative pressure was applied to the pipette to obtain a giga-ohm seal, brief suction was applied to the pipette to rupture the membrane, and then Vₑ and integrated firing rate were measured throughout the experiment. Integrated firing rate (Hz) was determined from the Vₑ trace in 10-s bins using a window discriminator (FHC high K⁺/nigericin technique (50). Rₐ values measured during the experiment were divided by the Rₐ value at pH 7.4 measured at the end of an experiment to yield values for normalized Rₐ (Nₐ). A calibration curve was used to convert unknown Nₐ values to pHₑ. The equation for this curve (Methods) was used to convert Nₐ values into pHₑ. B: pHₑ response of SC neurons from adult rats to an acidification induced by an NH₄Cl prepulse followed by either artificial cerebrospinal fluid (aCSF) (C) or 0 Na⁺ (D) solution. The control experiment (NH₄Cl prepulse followed by aCSF) exhibited recovery from NH₄Cl-induced acidification back to the initial pHₑ. In 0 Na⁺ solution, the neuron had a maintained acidification with pHₑ recovery inhibited. Upon replacing 0 Na⁺ solution with aCSF, pHₑ recovered rapidly back to initial pHₑ. 

Electrophysiological studies. The blind whole cell patch-clamp technique was used to measure neuronal membrane potential (Vₑ) and integrated firing rate, as described previously (1). The experimental setup that was used has been previously described (5, 8, 14, 18). Briefly, a whole cell patch pipette (5 MΩ) was fabricated from borosilicate glass using a Narishige PP-830 dual-stage pipette puller. The pipette was filled with whole cell patch solution (see above).
model 700B), which was then analyzed using pClamp 8.2 software. Viable neurons had a stable $V_m$ of between $-40$ and $-60$ mV and fired action potentials that crossed through zero. All recordings were done without current injection unless otherwise stated. In an attempt to minimize any sampling bias, we entered the SC slice at various locations with the blind patch pipette so as not to preferentially collect signals from neurons within one region. Further, since we could not visualize the neuron being patched, we simply obtained the first seal that we could successfully achieve and studied that neuron if it had a $V_m$ between $-40$ and $-60$ mV and had a spontaneous firing rate. This technique thus precluded any visually based sampling bias.

Sharp-tip recordings were used to assure that our whole cell recordings did not cause washout of the electrical response to HA as previously described (11, 22, 40). Sharp-tip electrodes were also fabricated from borosilicate glass, filled with 3 M potassium acetate solution, and were pulled to a tip resistance of $\sim 100-150$ M$\Omega$ using a one-stage Flaming/Brown micropipette puller (P87, Sutter Instruments, Novato, CA). Sharp-tip recordings began once a stable membrane potential and firing rate were reached.

Data analysis and statistics. To analyze $pH_i$, we averaged the last 5 $pH_i$ values (covering 5 min) before changing solution. To determine $pH_i$ recovery from acidification a $pH_i$ vs. time plot was made in Microsoft Excel, and the $pH_i$ recovery rate from CO$_2$-induced acidification of an individual neuron was estimated by the slope of a linear fit to the $pH_i$ vs. time trace during HA (at least five points starting at minimum $pH_i$). We recorded $V_m$ using Axoscope pCLAMP 8 (Axon Instruments, Sunnyvale, CA). Segments of at least 5 min of $V_m$ tracings were copied to MatLab software (MathWorks, Natick, MA) and $V_m$ was determined as the average of all values. The electrophysiological response to HA was quantified using two measures: percentage of neurons whose firing rate was reversibly altered by HA, and the magnitude of the response of SC neurons to HA was quantified by calculating the chemosensitivity index (CI) (53): $CI = 100\% \times \left(\frac{FR_{hc} - FR_{nc}}{FR_{nc}}\right)$, where $FR_{nc}$ is the firing rate at 15% CO$_2$, $FR_{hc}$ is the firing rate at 5% CO$_2$, $pH_{nc}$ is the $pH$ of aCSF, 7.45, and $pH_{hc}$ is the $pH$ of the HA solution, 6.85. A neuron was designated as activated if its CI was greater than 120% or inhibited if its CI was less than 80%. To determine the integrated firing rate of a neuron, we averaged all integrated firing rate values during exposure to hypercapnia starting 2 min after the start of the exposure. All values are reported as means ± SE. Fisher’s exact tests were used to compare differences between the percentages of neurons that respond to HA. To test for the difference of a mean from zero, we used a Student’s $t$-test for a single group mean. Paired $t$-tests were used to compare differences between two means with a level of significance at $P < 0.05$. Differences between three or more means were determined by one-way ANOVA. If significant differences existed, then multiple comparisons were done using Tukey’s method with a level of significance of $P < 0.05$. All $t$-tests and ANOVA were done using KyPlot 3.0 (Kyence, Tokyo, Japan).

**RESULTS**

$pH_i$ in SC neurons from adult rats. The steady-state $pH_i$ in SC neurons from adult rats is $7.30 \pm 0.03$ ($n = 27$), significantly ($P < 0.001$) less alkaline than the value of $7.49 \pm 0.02$ previously reported for SC neurons from neonatal rats (42). The NH$_4$Cl prepulse technique was used to study the $pH_i$ responses of SC neurons from adult rats. The normal response of $pH_i$ to an NH$_4$Cl prepulse is a rapid alkalinization followed by an acidification in the absence of any change in extracellular pH (2, 42). We found that the response to NH$_4$Cl of SC neurons from adult rats was similar to that of SC neurons from neonatal rats (41, 42). Adult SC neurons exhibited a rapid $pH_i$ recovery from an NH$_4$Cl prepulse induced acidification that returned toward the initial $pH_i$ (Fig. 1B, open squares). This recovery is entirely due to Na$^+$-dependent pH-regulating transporters (37) since it was reversibly inhibited by 0 Na$^+$ solutions (Fig. 1B, solid squares). These findings are consistent with Na$^+/H^+$ (NHE) exchange playing a predominant role in $pH_i$ recovery in SC neurons from adults, as it does in SC neurons from neonates (41, 42). However, we were not able to study $pH_i$ recovery more fully in adult SC neurons since the pH-transporter inhibitors amiloride and 4,4’-disothiocyanato-stilbene-2,2’-disulfonic acid (DIDS) interfered with pyranine fluorescence. DIDS and amiloride appeared to quench pyranine fluorescence upon excitation at 410 nm by as much as 20–30% and caused a dramatic shift in the fluorescence ratio.

$pH_i$ and electrophysiological responses to HA of SC neurons from adult rats in aCSF. A sample recording of the $pH_i$ and electrophysiological response to HA (15% CO$_2$) of an SC neuron from an adult rat is shown in Fig. 2. Each neuron was given a 5-min exposure to 5% CO$_2$, a 10-min exposure to 15% CO$_2$, and a final 5-min exposure to 5% CO$_2$. SC neurons from adult rats showed a similar $pH_i$ response ($P > 0.4$) to 15% CO$_2$ compared with SC neurons from neonatal rats, acidifying by 0.25 ± 0.011 pH unit in adults compared with 0.26 ± 0.006 pH unit in neonates (23). SC neurons from adult rats exhibited a lack of $pH_i$ recovery during HA ($-0.007 ± 0.002$ pH unit/min; $n = 69$). Upon return to normocapnia, the $pH_i$ of SC neurons returned to initial values with no apparent overshoot (Fig. 2), consistent with a lack of $pH_i$ recovery during HA (2, 42). Thus, SC neurons from adult rats exhibit a similar pattern of $pH_i$ recovery.
response to maintained HA as that previously reported for SC neurons from neonatal rats (42).

We also measured the membrane potential and integrated firing rate responses in parallel with pH, during HA in SC neurons. SC neurons were classified (on the basis of their integrated firing rate response to HA) as activated (CI > 120%), inhibited (CI < 80%), or nonchemosensitive (80% < CI < 120%). HA resulted in a significant (P < 0.001) depolarization of 3.7 ± 0.5 mV (n = 32) in SC neurons that were activated by HA, a nonsignificant (P > 0.05) depolarization of 5.1 ± 2.4 mV (n = 5) in inhibited neurons and a significant (P < 0.05) depolarization of 2.2 ± 1.0 mV (n = 19) in nonchemosensitive neurons. A sample trace of an activated SC neuron from an adult rat is shown in Fig. 2. We assessed neuronal responses to HA in two ways, determining the percentage of neurons that respond to and the magnitude of their neuronal responses to HA. We found that 57% of SC neurons from adult rats were activated (CI > 120%), 9% were inhibited (CI < 80%), and 34% were nonchemosensitive (n = 19). The average CI (magnitude of the chemosensitive response) for activated SC neurons from adult rats was 177 ± 8% and for inhibited SC neurons from adult rats was 63 ± 10%. Activated neurons in 5% CO2 had an average basal integrated firing rate of 0.6 ± 0.13 Hz that increased significantly (P < 0.001) to 2.0 ± 0.33 Hz in response to 15% CO2 and returned to initial values of 0.6 ± 0.12 Hz upon return to 5% CO2. Inhibited neurons had an integrated firing rate of 0.9 ± 0.30 Hz in 5% CO2, which fell to 0.3 ± 0.08 Hz in response to 15% CO2 (the fall was not significant due to large variations in initial firing rate, but all 5 neurons had a greater than 20% fall of CI) and returned to 0.5 ± 0.24 Hz upon return to 5% CO2. Nonchemosensitive neurons had an integrated firing rate of 1.1 ± 0.42 Hz in 5% CO2, which did not change significantly (P > 0.5) in 15% CO2 (1.0 ± 0.30 Hz), and remain unchanged upon return to 5% CO2 (0.5 ± 0.11 Hz).

We used sharp-tip recordings to see whether our whole cell recordings caused alterations of the electrical response of these neurons to HA. It was found that 49% of SC neurons from adult rats were activated (21/43) with a CI of 133 ± 5%, while none were inhibited (data not shown). In the activated neurons, firing rate was 1.5 ± 0.22 Hz and increased significantly (P < 0.001) to 2.8 ± 0.37 Hz (n = 21) in response to HA. The percentage activated with sharp-tip recordings (49%) is not significantly (P > 0.425) different than the findings from the whole cell recordings (57%). However, the CI of SC neurons activated by HA recorded using sharp-tip electrodes (133 ± 5%) is significantly (P < 0.001) lower than the CI found for activated SC neurons using the whole cell pipettes (177 ± 8%). It is not clear why the CI is higher for activated SC neurons using the whole cell technique compared with the sharp-tip technique, but it may be due to the somewhat higher initial firing rate measured with sharp-tip vs. whole cell pipettes, which has been shown to affect the calculated values of CI (19). We note that the absolute increase in firing rate induced by HA is nearly identical when measured with whole cell (1.4 Hz) vs. sharp-tip (1.3 Hz) pipettes. Nevertheless, it is clear that using our modified whole cell solution (15) prevents the washout of the firing rate response to HA that was previously seen in adult SC neurons (11).

\( pH_1 \) and electrophysiological responses to HA of SC neurons from adult rats in SNB and SNB + CARB. We next wanted to measure the response to HA, in the presence of chemical and electrical synaptic blockade, of SC neurons from adult rats. Thus, we first blocked chemical transmission using SNB. SNB solution certainly reduces vesicular neurotransmitter release, but it likely does not inhibit any nonvesicular neurotransmitter release that may occur in NTS neurons. Nevertheless, the effectiveness of SNB in blocking vesicular neurotransmitter release is suggested by the elimination of spontaneous postsynaptic potentials in the presence of SNB (Fig. 3A, inset, and Fig. 3B). A sample recording of the basal firing rate response to SNB is seen in Fig. 3A, which shows that basal firing rate increases in response to SNB. The average basal firing rate in aCSF of SC neurons from adult rats (n = 14) increases significantly (P < 0.05) when medium is changed from aCSF to SNB (0.7 ± 0.22 Hz vs. 1.6 ± 0.5 Hz, respectively) (Fig. 3C).

-associated with the increased firing rate, SNB resulted in
We determined the effect of SNB on SC neuronal responses to HA. To compare HA-induced effects on firing rate, we wanted to start from nearly the same initial firing rate as in aCSF. Thus, in the presence of SNB, before the neuron was exposed to HA, the firing rate was decreased back toward initial firing rate by injecting negative DC current (Fig. 4, hyp). It was found that SNB did not significantly (P > 0.05) affect the magnitude of acidification caused by HA (0.21 ± 0.02 pH units in the presence of aCSF and 0.15 ± 0.03 pH units in the presence of SNB) (n = 18). Additionally, there was still a lack of pH_{i} recovery from HA-induced acidification in the presence of SNB (−0.015 ± 0.003 pH unit/min in aCSF and −0.006 ± 0.003 pH unit/min in the presence of SNB) (Fig. 4). The integrated firing rate response to HA in SC neurons from adult rats also did not change in the presence of SNB. Fig. 4 shows a sample trace of an HA-activated SC neuron from an adult rat in the presence of SNB. Details of the firing rate response to HA in SNB of SC neurons from adult rats will be given below.

We also studied the response to HA of SC neurons from adult rats in solutions in which both chemical synaptic transmission (SNB) was blocked and electrical transmission was blocked (using carbamolone, CARB). Fig. 5 shows a sample pH_{i} and electrophysiological response to HA in the presence of SNB + CARB from an HA-activated SC neuron. Before the HA response was measured in the presence of SNB + CARB, the firing rate was decreased back toward initial firing rate by injecting negative DC current. We found that the acidification induced by HA was significantly larger in the presence of SNB + CARB (0.52 ± 0.06 pH unit in the presence of SNB + CARB; n = 17) (P < 0.001). There was still a lack of pH_{i} recovery in response to HA in SNB + CARB in SC neurons from adult rats (−0.011 ± 0.005 pH unit/min) (Fig. 5). Upon return to normocapnia, the pH_{i} of SC neurons returned to initial values with no apparent overshoot (Fig. 5), consistent with a lack of pH_{i} recovery during HA (2.42). The integrated firing rate response to HA in SC neurons from adult rats did not change in the presence of SNB + CARB (n = 16).

Fig. 6A summarizes the percentage of SC neurons from adult rats that were activated or inhibited by HA in aCSF (open bar), SNB (gray bar), or SNB + CARB (black bar). We found that SNB by itself (only chemical transmission blocked; 57%) and SNB + CARB (both chemical and electrical transmission; 56%) had no effect (P > 0.05) on the percentage of SC neurons from adult rats that were activated by HA (aCSF = 57%) or on the percentage of SC neurons from adult rats that were inhibited by HA (Fig. 6A). Under all conditions, relatively few (10–20%) SC neurons from adult rats are inhibited by HA. Just as in the absence of SNB and CARB, in the presence of SNB + CARB HA resulted in a significant (P < 0.01) depolarization (7.6 ± 2.1 mV; n = 9) of activated SC neurons, but no significant (P > 0.05) change of membrane potential in inhibited (3.3 ± 2.6 mV hyperpolarization; n = 3) or nonchemosensitive (1.0 ± 1.0 mV; n = 4) SC neurons. Interestingly, the HA-induced depolarization in activated SC neurons was significantly (P < 0.01) greater in the presence of SNB and CARB (7.6 mV) than in aCSF alone (3.7 mV). Thus, in terms of changes of membrane potential and percentages of SC neurons from adults that respond to HA, the responses measured in aCSF appear to be intrinsic responses and not dependent on chemical or electrical synaptic transmission.

Fig. 6B summarizes the magnitude of the response to HA (determined as CI) in activated SC neurons from adult rats in aCSF (open bar), SNB (gray bar), and SNB + CARB (black bar). We found that the CI of activated SC neurons in aCSF (177 ± 8%) was the same (P > 0.05) as the CI in SNB (162 ± 14%) and in SNB + CARB (189 ± 18%). In summary, in adult rats, both with respect to the magnitude and the percentage of SC neurons that respond to HA, the responses seen in aCSF appear to be intrinsic and not dependent on chemical or electrical synaptic input.

pH_{i} and electrophysiological responses to isohydric hypercapnia of SC neurons from adult rats. Last, we measured the pH_{i} and electrophysiological responses to IH of SC neurons from adult rats. A sample record of the pH_{i} and electrophysiological response to IH from an SC neuron from an adult rat is shown in Fig. 7. All experiments with IH were done in the presence of SNB throughout. SC neurons from adult rats exhibited a similar pattern of pH_{i} response to IH as previously reported for SC neurons and LC neurons from neonatal rats.
This includes a small IH-induced acidification followed by \( \text{pH}_i \) recovery, with \( \text{pH}_i \) exhibiting an alkaline overshoot upon return to normocapnia. This was accompanied by a reversible increase in firing rate induced by IH (Fig. 7). On average, activated SC neurons from adult rats showed a significantly \((P < 0.001)\) smaller \( \text{pH}_i \) response to IH \((0.11 \pm 0.03; n = 10)\) than SC neurons from adult rats in response to HA \((0.21 \pm 0.04; n = 7)\) (Fig. 8A). IH-induced \( \text{pH}_i \) recovery was evident in 13/19 SC neurons from adult rats \((0.011 \pm 0.002 \text{ pH unit/min})\) with 6 neurons having no \( \text{pH}_i \) recovery from IH-induced acidosis (Fig. 8B). This response is very different from the \( \text{pH}_i \) response of SC neurons from adult rats to HA, where only 4/19 neurons showed at most a slight recovery \((0.003 \pm 0.004 \text{ pH unit/min})\) (Fig. 8B).

We could not assess the magnitude of neuronal firing rate responses to IH using CI as CI values are normalized to the change in \( \text{pH}_i \), and \( \text{pH}_i \) does not change with IH. Thus, we quantified the neuronal response to IH by measuring the change in firing rate that is induced by both HA and IH. IH induced an increase in firing rate from \(1.3 \pm 0.21 \text{ Hz}\) to \(4.4 \pm 0.57 \text{ Hz} (n = 11)\) \((P < 0.001)\) and HA induced an increase in firing rate from \(0.5 \pm 0.12 \text{ Hz}\) to \(3.0 \pm 0.96 \text{ Hz} (n = 8)\) \((P < 0.05)\). The increases in firing rate induced by IH \((3.1 \pm 0.51 \text{ Hz})\) and HA \((2.5 \pm 0.86 \text{ Hz})\) were not different \((P > 0.5)\) for activated SC neurons from adult rats (Fig. 8C). We also looked at the percentage of SC neurons that responded to the two conditions. It was found that 54% of SC neurons from adult rats were activated in HA \((n = 8/15)\) and 73% were activated in IH \((n = 11/15)\), while 13% were inhibited in both cases \((n = 2/15)\). There was no statistical difference \((P > 0.4)\) either for the percentage activated or the percentage inhibited by HA or IH. Unlike HA, IH resulted in no significant \((P > 0.05)\) membrane potential change in activated \((0 \pm 1.6 \text{ mV}; n = 11)\) SC neurons. There was no evident depolarization with IH in HA-inhibited or nonchemosensitive SC neurons either, but with only two neurons of each type, we could not determine a standard error and test for significance.

It should be noted that upon exposure to IH, there is no change in \( V_m \), and hypercapnia induces a transient acidification. Nevertheless, firing rate increases and continues to increase even while \( \text{pH}_i \) is exhibiting alkaline recovery (Fig. 7, representative of over half of the records). These observations clearly show that the \( V_m \) responses to hypercapnia are complex and that a change of \( \text{pH}_i \) alone is not solely responsible for chemosensitive signaling, supporting the concept of multiple factors in chemosensitive signaling (38).

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**Fig. 5.** The \( \text{pH}_i \) and firing rate response of an individual SC neuron from an adult rat that was activated by hypercapnic acidosis (HA) in the presence of SNB + CARB. 

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**Fig. 6.** A: summary of the percentage of SC neurons from adult rats that respond to hypercapnic acidosis (HA). We found that the % activated in the presence of only aCSF (open bar) was not changed by SNB (gray bar) or SNB + CARB (solid bar). The percentage of inhibited neurons (9% in aCSF) was similarly unaffected by SNB (7%) and by SNB + CARB (19%). N values are denoted on each bar and height of bars represent the mean values. B: summary of the chemosensitivity index (CI) of SC neurons from adult rats that are activated in response to HA. We found that the CI of activated SC neurons from adult rats in the presence of aCSF only (white bar) was not affected by SNB (gray bar) or SNB + CARB (black bar). Height of bar represents mean CI, and error bar represents 1 SE.
DISCUSSION

We studied the cellular properties of SC neurons from adult rats and their intrinsic responses to hypercapnia. The main findings of this study are that compared with SC neurons from neonatal rats, SC neurons from adult rats 1) have a lower initial pHi; 2) have similar responses to IH (reduced acidification, pHi recovery, and increased firing rate); and 3) have a similar intrinsic firing rate response to HA. Thus, the chemosensitive response of SC neurons from adult rats is similar to the chemosensitive response of SC neurons from neonatal rats (5), indicating that the chemosensitive response of adult SC neurons is established early in neonatal development.

$pHi$ responses of SC neurons from adult rats. We found that SC neurons from adult rats have a steady-state pHi ($\text{pH}_i$) that is about 0.2 pH unit lower than SC neurons from neonatal rats (42). In neonates, steady-state pHi of SC neurons is largely determined by alkalinizing Na$^+$/H$^+$ exchange (NHE) (41). A major role for NHE exchange in pHi regulation in SC neurons from adults is consistent with our finding that all recovery from acidification is Na$^+$/H$^+$ dependent (Fig. 1B), although we cannot rule out a contribution from Na$^+$/H$^+$-driven HCO$_3^-$-dependent transporters (37). SC neurons from neonates are unique among the medullary neurons studied to date in that they largely lack acidifying Cl$^-$/HCO$_3^-$ exchange. Using our experimental approach, we were not able to assess the possible role of HCO$_3^-$-dependent transporters since the commonly used inhibitors (amiloride and DIDS) quenched pyranine fluorescence at 410-nm excitation. The role of HCO$_3^-$ transporters in pHi regulation in SC neurons from adults is being determined by an ongoing study using 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescin.

Responses to HA and IH of SC neurons from adult rats. Studies of the pHi response to hypercapnic acidosis (HA; 15% CO$_2$, pH$_o$ 6.8–6.9) in SC neurons from neonatal rats have could result if adult neurons had increased expression of Cl$^-$/HCO$_3^-$ exchange. Using our experimental approach, we were not able to assess the possible role of HCO$_3^-$-dependent transporters since the commonly used inhibitors (amiloride and DIDS) quenched pyranine fluorescence at 410-nm excitation. The role of HCO$_3^-$ transporters in pHi regulation in SC neurons from adults is being determined by an ongoing study using 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescin.

Fig. 7. The pHi and firing rate response of an individual SC neuron from an adult rat that was activated by isohydric hypercapnia (IH) in the presence of SNB. A: experimental protocol used. B: pH$_i$ response to IH over time of the SC neuron. Notice that there is pH$_i$ recovery during IH and that once the IH solution was removed, there was an alkaline overshoot in pH$_i$ that returned back to a value above initial pH$_i$. C: firing rate response to IH over time of the SC neuron, which increased reversibly in response to IH. D: 10-s samples of action potentials taken at the time points indicated in C. Notice that action potential frequency increased with IH and then decreased back to initial frequency once the IH solution was removed.

pHi responses of SC neurons from adult rats. We found that SC neurons from adult rats have a steady-state pHi ($\text{pH}_i$) that is about 0.2 pH unit lower than SC neurons from neonatal rats (42). In neonates, steady-state pHi of SC neurons is largely determined by alkalinizing Na$^+$/H$^+$ exchange (NHE) (41). A major role for NHE exchange in pHi regulation in SC neurons from adults is consistent with our finding that all recovery from acidification is Na$^+$/H$^+$ dependent (Fig. 1B), although we cannot rule out a contribution from Na$^+$/H$^+$-driven HCO$_3^-$-dependent transporters (37). SC neurons from neonates are unique among the medullary neurons studied to date in that they largely lack acidifying Cl$^-$/HCO$_3^-$ exchange (41), which seems to account for their very alkaline values of pH$_i$. The lower steady-state value of pH$_i$ in SC neurons from adults...
found a ΔpH$_i$ of 0.26 ± 0.006 pH unit (23) in response to HA and a maintained acidification with a lack of pH$_i$ recovery from that acidification (42), as seen in other chemosensitive neurons (3, 31, 55). There is no recovery during the HA challenge because NHE, the predominant pH$_i$ recovery mechanism following acidification in medullary neurons (37, 42), is inhibited by the extracellular acidification associated with HA (41). To our knowledge, there are no studies looking at the pH$_i$ response to HA in individual SC neurons from adult rats. In the current study, we found that the acidification induced by HA was 0.25 ± 0.011 pH unit, which is almost identical to what was seen in neonates (23). We also found that SC neurons from adult rats lacked pH$_i$ recovery during the HA exposure (Fig. 2). This lack of pH$_i$ recovery was also seen in neurons from neonates (42). We conclude that the pH$_i$ response to HA in adult rats is similar to neonates and thus does not change during development.

The electrical response to HA has been studied in caudal SC neurons from neonatal rats. In neonates, about 50% of caudal SC neurons are activated with a CI of about 150%, and about 10–15% are inhibited with a CI of 57 ± 6% (5). Studies done on SC neurons from adult rats have only looked at the percentage of neurons that were activated by HA (7, 10, 21, 22). In a mix of caudal and more rostral slices, 35% of SC neurons from adult rats were found to be activated in response to HA (7), while in caudal slices only, 53% of SC neurons from adult rats were activated in response to HA (22). The current study examined SC neurons exclusively taken from the caudal region of the SC from adult rats. We found very similar results to the results from the neonatal studies (5) and the results from Mulkey et al. (22) in that 57% were activated with a CI of 177 ± 8% and 9% were inhibited with a CI of 63 ± 10%. The similar percentages of activated and inhibited SC neurons using a variety of techniques suggest that we are randomly sampling SC neurons, or else all techniques (perforated patch, whole cell patch, sharp tipped electrodes) have a similar bias, which seems unlikely. We also report here that HA induces a significant depolarization in SC neurons that are activated but not in inhibited SC neurons. We conclude that the electrical response to HA of SC neurons from adult rats is quite similar to that of SC neurons from neonates, implying that the electrical response of SC neurons to HA also does not appear to change appreciably during development. However, while the change of pH$_i$ and the increase in firing rate in response to HA are the same in neonates and adults, adults have a lower initial pH$_i$ than neonates. To the degree that changes of pH$_i$ determine the firing rate response to HA, these findings imply that there has been a shift in pH$_i$ sensitivity in adult SC neurons to a lower value of pH$_i$ compared with neonates.

We also studied the response of SC neurons from adult rats to a different hypercapnic stimulus, IH, in which pH$_o$ is maintained constant. The response to IH has been studied in SC neurons from neonatal rats (42). IH causes a smaller acidification than HA and unlike the lack of pH$_i$ recovery seen during HA, there was pH$_i$ recovery during the IH exposure. When IH was removed, SC neurons exhibited an alkalinizing overshoot (42). We found that the pH$_i$ response to IH of SC neurons from adults was similar to the response of neonates, namely, a smaller acidification, during IH, and an alkaline overshoot upon return to aCSF (Fig. 7). The presence of pH$_i$ recovery with IH-induced acidification but not with HA-induced acidification suggests that as with neonates, pH$_i$ recovery mechanism(s) are present in SC neurons from adult rats, but that these mechanism(s) are inhibited by extracellular acidification (41, 42).

SC neurons from adult rats increase their firing rate in response to IH (Fig. 7). We have not studied the firing rate response to IH for SC neurons from neonatal rats, but it is instructive to compare the responses to IH vs. HA for SC neurons from adults. HA results in a significant membrane depolarization (~4 mV) in HA-activated SC neurons and a large change in pH$_i$ (~0.25 pH unit), whereas IH does not cause a membrane depolarization and induces a smaller change in pH$_i$ (~0.11 pH unit) in SC neurons from adult rats (Fig. 8A). Despite these differences, the increase in firing rate induced by HA and IH in SC neurons is similar (Fig. 8C). This pattern of IH inducing an increased firing rate without a membrane depolarization has previously been seen in LC neurons (14) and a pattern of a smaller ΔpH$_i$ with IH than with HA but a similar firing rate response to the two acid challenges has been observed in neurons from the LC (14) and the RTN (44). Further, the maximal firing rate upon exposure to IH occurred when pH$_i$ was alkaline (Fig. 7). Thus, while IH-induced acidification may initiate increased firing rate, other factors (possibly including increased CO$_2$ and/or increased intracellular calcium) must also be involved in chemosensitive signaling (38). Interestingly, it has been shown that there is not a unique relationship between the fall of pH$_i$ and increased ventilation (13). Finally, HA is able to stimulate the firing rate of LC neurons even when pH$_i$ is unchanged (17). These data clearly indicate that the magnitude of the fall of pH$_i$ (and/or pH$_o$) is not the sole determinant of the firing rate response of chemosensitive neurons to acid challenges. In fact, in neurons from all brain stem regions of juvenile and adult rats, there is a lack of pH$_i$ recovery from HA (31), further indicating that a change of pH$_i$ is not the determining factor in chemosensitive neurons, suggesting that chemosensitive signaling involves multiple factors (38).

Intrinsic chemosensitivity of SC neurons from adult rats. A major concern with our previous measurements of the HA response of SC neurons in slices bathed in aCSF is whether the neuron that we are studying is intrinsically chemosensitive or not. The term “intrinsic” has been used with various meanings. Chemosensitive neurons have been claimed to be intrinsic based on the elimination of vesicular chemical neurotransmitter release (7, 24, 32, 40) using either high Mg$^{2+}$/low Ca$^{2+}$ solutions or receptor-blocking cocktails. More recent evidence shows that gap junctions are expressed in neurons from chemosensitive regions (9, 46, 48) and are preferentially expressed in CO$_2$-sensitive neurons from the NTS (8, 18). We have thus recently (5), and in the current study, extended the definition of an intrinsically chemosensitive neuron to one that still responds to hypercapnia when both vesicular chemical neurotransmitter release and electrical synaptic activity are blocked. There are nonvesicular pathways of neurotransmitter release, and electrical synaptic activity are blocked. There are nonvesicular pathways of neurotransmitter release, and electrical synaptic activity are blocked. There are nonvesicular pathways of neurotransmitter release, and electrical synaptic activity are blocked. There are nonvesicular pathways of neurotransmitter release, and electrical synaptic activity are blocked.
hypercapnia when vesicular chemical neurotransmitter release and gap junctions are blocked.

It is possible that a neuron increases its firing rate in response to HA due to increased excitatory chemical synaptic input or through direct electrical coupling with another neuron that is intrinsically responding to HA. The effects of vesicular chemical synaptic transmission have been assessed in SC neurons from both neonates (5, 18) and adults (7) using SNB. SNB alone has been shown to decrease the initial spontaneous firing rate of SC neurons from both neonates (5) and adults (7). In the current study with adults, however, we found that SNB results in a marked depolarization and an increase in spontaneous firing rate (Fig. 3). The differences in these findings with the previous studies in adults may be due to the inclusion of more rostral slices in the earlier studies. It may be that the local circuit differs in the caudal SC vs. the more rostral SC. Further, the finding of an SNB-induced decrease in firing rate by Dean et al. (7), but an SNB-induced increase in firing rate in the present study may be due to the use of dorsal medullary slices (with the ventral part of the slice removed) by Dean et al. (7). It is possible in our study that the inhibitory input arises from the ventral portion of the slice and would thus not be evident with the preparation used by Dean et al. (7). Finally, the increased SC firing rate in SNB medium suggests that in adult medullary slices the input to the SC is, on balance, inhibitory. This is consistent with GABergic neurons spread throughout the NTS, as well as with GABergic projections to the NTS from the intermedius nucleus of the medulla, a region included in the medullary slices that we used (12). Our findings thus suggest that in intact medullary slices, SC neurons from neonates receive, on balance, excitatory input, while SC neurons from adults receive inhibitory input.

SNB has little effect on the response to HA of SC neurons from rats of all ages. SNB was shown not to alter the pH, response to HA of SC neurons from neonates (20), and we found here no effect in SC neurons from adult rats. Both the magnitude and percentage of the firing rate responses to HA were similarly largely unaffected by SNB in SC neurons from neonates (5), although Huang et al. (18), using more rostral slices, found that SNB reduced the percentage of activated SC neurons from 34% to 25% (18). In adults, we find no effect of SNB on the pH, and firing rate response of SC neurons to HA (Fig. 6, A and B), which agree with previous findings that SNB reduced the percentage of adult SC neurons that are activated by HA by at most 10% (7). Thus, it seems that the excitatory response of the vast majority of chemosensitive neurons to HA does not require synaptic input in SC neurons from rats of all ages.

In the current study, we also measured the chemosensitive response of caudal SC neurons from adult rats in the presence of SNB + CARB to remove electrical, as well as chemical synaptic transmission. Chemosensitive SC neurons from neonates are preferentially gap junction coupled (7, 18) and connexins (the proteins that form gap junctions) are expressed in SC neurons from both neonates and adults (46, 48). Carbamoloxone has been shown to effectively uncouple chemosensitive neurons (9) and is used widely to block gap junctions (9, 34, 39), although it is known to have other effects (36, 45, 51). We found that HA resulted in a significantly larger membrane depolarization in HA-activated SC neurons from adults in the presence of SNB + CARB (~4 mV) than in aCSF only (~4 mV). This may be due to a higher input resistance in neurons without synaptic input and with gap junctions blocked. Surprisingly, we also found that the HA-induced intracellular acidification was significantly larger in SNB + CARB (Fig. 5) in SC neurons from adults, although we previously reported no differences in SC neurons from neonates (20). This effect of carbamoloxone on pH, is similar to the effect of gap-junction blockers on pH, in astrocytes (33). In astrocytes, the acidification was larger in the presence of HCO_3^- and diminished by HCO_3^- transport blockers, suggesting that gap junction blockers alter the activities of pH-regulating transporters, especially Na-HCO_3^- cotransport (33). While no HCO_3^- dependent transporters have been found in SC neurons from neonates (41), there is some evidence that they are present in SC neurons from adults (Martino PF and Putnam RW, unpublished observations). It is noteworthy that the increased membrane depolarization and the increased acidification induced by HA in the presence of SNB + CARB are not accompanied by a larger firing rate response, further indicating the lack of a simple relationship between V_m, pH, and firing rate in SC neurons (38), and suggesting that the response of chemosensitive neurons to hypercapnia may have a maximal response (35, 43). Regardless, in SC neurons from adult rats, neither the magnitude nor the percentage of neurons responding to HA was affected by SNB + CARB. In SC neurons from neonates, the percentage of SC neurons that were activated by HA was decreased by CARB in rats aged P1–P10, but carbamoloxone had no effect in older neonates (5, 29). These findings suggest that except in very young neonates, the response of SC neurons to HA is intrinsic and does not rely on chemical or electrical synaptic input.

**Perspectives and Significance**

This is the first extensive study of intrinsic chemosensitivity in SC neurons from adult rats. The key finding of this study combined with an earlier study on SC neurons from neonatal rats (5) is that after about neonatal day P10, chemosensitive SC neurons are fully developed and express an intrinsic response to HA. This is important for studying the basis of cellular chemosensitive signaling, which requires intrinsically chemosensitive neurons. We have recently performed a similar analysis of the early development of LC neurons, another putative chemosensitive region (16). These studies show a high percentage (60–80%) of intrinsic chemosensitivity among catecholaminergic LC neurons from rats younger than P10, but after that the percentage of intrinsically chemosensitive neurons falls sharply to only about 20% in rats aged P15–P19. Adrenal chromaffin cells also respond to hypercapnia with a release of catecholamines, and this response is strongest in young neonates and decreases markedly in rats up to about age P10 (25). Finally, serotonergic neurons from the medullary raphé showed an increase in both the percentage of neurons that are activated by hypercapnia and the magnitude of that response in neurons from rats older than P12 compared with neurons from younger rats, whether the neurons were in brain slices or in tissue culture (53). It is instructive to compare this pattern of early maturity of chemosensitivity in putative chemosensitive neurons with the early developmental pattern of the whole animal ventilatory response to inspired CO_2. The exact pattern of this development is uncertain, being either
triphasic with a minimum point at about P5–P10 (49) or involving a dramatic increase in the hypercapnic ventilatory response from P15 to P20 (6). Both of these patterns show an increase in the ventilatory response to hypercapnia between P10 and P15 that agrees with the development of chemosensitivity in serotonergic medullary raphé neurons (53). A significant ventilatory response to hypercapnia in young neonates (P1–P5) may suggest an involvement of chemosensitive LC neurons (16) and/or adrenal chromaffin cells (25). Although the developmental pattern of cellular chemosensitivity in SC neurons does not follow either developmental pattern of the ventilatory response to hypercapnia, we do not rule out a role for chemosensitive SC neurons in ventilatory control. Because chemosensitivity is a distributed property involving numerous chemoreceptive sites (28), it is likely that other sites play an important role in early development, both centrally and peripherally. It is also possible that the interaction of chemosensitive neurons from various regions within the respiratory network will be crucial for evaluating the last possibility.

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